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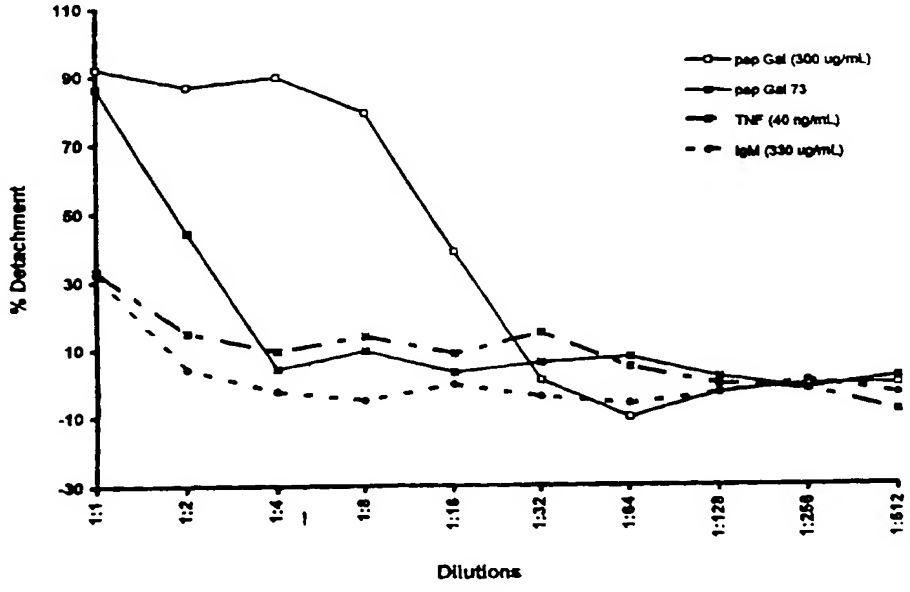
(54) Title: HUMAN SERUM LECTIN - INDUCED APOPTOSIS AND METHOD FOR DETECTING APOPTOSIS

(57) Abstract

The unifying theme of the present invention is the description of a class of proteins present in human blood which have the capacity to selectively recognize and induce apoptosis of cells which convey abnormal surface carbohydrates. Alterations in cellular glycosylation can result from either species-specific differences or may reflect cancer-associated changes. Human serum carbohydrate binding proteins, referred to as lectins, were isolated on several different carbohydrate columns including Gal alpha (papGal), GalNAc, GlcNAc, Fucose, Mannose and Melibiose. These lectins were shown to cause apoptosis of porcine aortic endothelial cells. This demonstrates a new pathway leading to endothelial cell death following xenogenic stress that may be important in the pathogenesis of xenograft rejection and suggests new therapeutic approaches to the treatment of xenograft rejection. Further, these mammalian carbohydrate binding proteins are herein shown to cause apoptosis of breast cancer and lung cancer cells *in vitro*, and can be used as a cytotoxic agent against human tumour cells. Significantly, the proteins of the present invention exhibit minimal binding or reactivity against normally glycosylated human cells. This invention also relates to a novel bioassay for the detection and quantitation of cellular apoptosis *in vitro*, and to the utility of this assay for the identification and evaluation of compounds with potential apogenic activity.



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# HUMAN SERUM LECTIN - INDUCED APOPTOSIS AND METHOD FOR DETECTING APOPTOSIS

## FIELD OF INVENTION

The present invention relates to a new type of human serum lectins which have the property of inducing apoptosis in certain cell types. This invention also relates to a method of using said lectins to induce apoptosis, specifically, to induce apoptosis in transformed and malignant cells. Conversely, inhibition of said lectins in order to inhibit apoptosis is beneficial in some circumstances, such as xenotransplantation, and methods to achieve this are described in the present invention. This invention also relates to a novel bioassay for the detection and quantitation of cellular apoptosis *in vitro*, and to the utility of this assay for the identification and evaluation of compounds with potential apogenic activity.

## BACKGROUND OF THE INVENTION

### Apoptosis

Apoptosis, or programmed cell death, is a common mechanism of cell elimination in the multicellular organism. It occurs during embryonic development, and in a variety of normal and malignant adult tissues. Apoptosis can be induced by various signals, such as exposure to corticosteroids, cytotoxic T lymphocytes, tumour necrosis factor alpha (TNF alpha), certain monoclonal antibodies (mAb) and cytotoxic drugs. In addition, apoptosis may result from the absence of signalling, such as the deprivation of growth hormone (Arends M., et al., Am. J. Pathol., 136:593-607 (1990); Cotter T., et al., Anticancer Res., 10:1153-1160(1990); Gerschenson L. and Rotello R., FASEB J., 6:2450-2455 (1992)). The precise biochemical events leading to apoptosis remain obscure. Recently, several genes have been identified that are involved in the regulation of programmed cell death (Henderson S., et al., Cell, 65: 1107-1115

(1991); Yonish-Rouach E., et al., Nature, 353: 345-347 (1991)).

Lectins

Lectins are proteins or glycoproteins that specifically and reversibly bind to carbohydrates (Sharon N. and Lis H., Science, 246: 227-234 (1989)). Mammalian lectins appear to be ubiquitous on the surface of normal as well as abnormal cell types, and although characterized by great structural diversity, are functionally unified by their capacity to bind to endogenous carbohydrate ligands. For descriptive purposes, the carbohydrate-binding proteins which cause apoptosis and are described in the present invention are referred to as "apogens" or "lectins," recognizing that they are distinct from previous structures given in the following description. Classically, two main classes of lectins have been described: C-type lectins, having a Ca2+-dependent carbohydrate binding activity and containing a distinct carbohydrate recognition domain; and S-type lectins, which typically display a specific affinity for beta-galactoside residues. Mannan-binding protein (MBP) is a prototypical calcium-dependent lectin postulated to play a role in innate defence against microorganisms by virtue of its capacity to activate the classical pathway of the complement system. The classification and functional properties of lectins have been reviewed (Harrison F.L., Journal of Cell Science, 100:9-14 (1991); Ryley N.G., et al., Immunological Methods, 141: 73-79 (1991)), including newly recognized I-type lectins representing the evolutionarily primitive immunoglobulin superfamily (IgSF) (Powell L.D. and Varki A., J Biological Chemistry, 270: 14243-14246 (1995)). Although some plant lectins, such as phytohaemagglutinin (PHA), concanavalin A (ConA), wheat germ agglutinin (WGA), and Lens culinaris (LCA), are cytotoxic (Stanley P., The Biochemistry of Glycoproteins and Proteoglycans, pp. 161-190 (1981)), no previous description exists regarding the apogenic capacity of mammalian serum lectins towards cancer cells.

Xenogenic Apoptosis

Transplantation of animal organs into humans, or xenotransplantation, offers a solution for the critical shortage of human organs presently available for conventional transplantation. Transplantation across discordant species barriers, such as in the porcine-to-primate combination, is invariably complicated by the process of xenograft rejection. Xenograft rejection is a fulminant response resulting in widespread graft thrombosis and rapid loss of graft function (Kaufman C.L., et al. Annual Review of Immunology, 13: 339-67 (1995); Hammer C., et al., Pathologie Biologie, 42(3): 203-7 (1994); Makowka L., et al., In: Hackel E., AuBuchon J., eds. Advances in Transplantation. Behesda MD: American Association of Blood Banks, 1993: 93-112; and Platt J.L., et al., Transplantation, 52(6): 937-47 (1991)).

In vascular grafts, the first target of rejection is the vascular endothelium, which is essential for maintenance of homeostasis. In the clinically applicable porcine-to-primate model, graft rejection is conventionally thought to be initiated by preformed antibodies binding to xenoantigens expressed on porcine endothelium, leading to endothelial cell dysfunction manifested as heparin sulfate release, transcription of activation proteins, and the induction of a procoagulant state (Bach F.H., et al., Immunological Reviews, 141: 5-30 (1994); Gerritsen M.E., et al., Faseb Journal, 7(6): 523-32 (1993); Platt J.L., et al., Journal of Experimental Medicine, 171(4): 1363-8 (1990)). Endothelial cell activation may result from the activation of complement, since it is commonly held that porcine complement inhibitors are species specific and are relatively unable to inhibit human complement activation (Dalmasso A.P., et al., Clinical and Experimental Immunology, 1: 31-5 (1991); Dalmasso A.P., Immunopharmacology, 24(2): 149-60 (1992)). Complement activation may produce endothelial cell membrane damage as a result of terminal attack complex formation (Kennedy S.P., et al., Transplantation, 57(10): 1494-501 (1994)). The pathological correlates of these events include antibody deposition, thrombosis, complement deposition, endothelial cell disruption, and cellular necrosis (Lexer G., et al., Journal of Heart Transplantation, 5(6): 411-18

(1986)).

We show for the first time in the present invention that a xenogenic environment can induce endothelial cell apoptosis, and we speculate that this phenomenon, previously described in other pathological states, may account for many of the histopathological features of xenograft rejection. We have shown that apoptosis occurs in an *in vivo* model of xenograft rejection. Apoptosis has been described as a physiological form of cell death, which differs from necrosis, or accidental cell death, in both biochemical mechanisms and pathological appearance (Ueda N., et al., Journal of Laboratory and Clinical Medicine, 124(2): 169-77 (1994); Eastman A., Toxicological and Applied Pharmacology, 121(1): 160-4 (1993)). Endothelial cell apoptosis can be triggered by TNF alpha (Robaye B., et al., American Journal of Pathology, 138(2): 447-53 (1991); Polunovsky V.A., et al., Experimental Cell Research, 214(2): 584-94 (1994), lipopolysaccharide, heat shock (Buchman T.G., et al., American Journal of Physiology, 265 (1 Pt 2): H165-70 (1993)), growth factor deprivation (Araki S., et al., Biochemical and Biophysical Research Communications, 172(3): 1081-5 (1990); Araki S., et al., [published erratum appears in Biochem Biophys Res Commun, 169(3): 1248 (1990)]), inhibition of cellular attachment (Re F., et al., Journal of Cell Biology, 127(2): 537-46 (1994); Meredith J.Jr., et al., Molecular Biology of the Cell, 4(9): 953-61 (1993)), and other stimuli (Haimovitz-Friedman A., et al., Journal of Experimental Medicine, 180(2): 525-35 (1994); Araki S., et al., Biochemical and Biophysical Research Communications, 190(1): 148-53 (1993); Wendt C.H., et al., American Journal of Physiology, 267 (4 Pt 1): C893-900 (1994)).

Thus, one aspect of the present invention is directed to human serum apogens, and more specifically, to human serum lectins which have been found to induce apoptosis of xenogenic cells. Various methods can be used to inhibit the actions of such lectins in order to inhibit or reduce apoptosis and thus would be useful for the reduction or prevention of xenograft rejection. In this invention xenogenic cells include but are not limited to: sheep cells, mouse cells, non-human primate cells, kangaroo cells, ostrich cells and porcine cells.

Lectin-mediated Apoptosis of Cancer Cells

Apoptotic cell death is important in several physiologic and pathologic processes and appears to be intrinsically programmed. An emerging concept of considerable importance holds that apoptosis is a physiologically regulated process which not only opposes unregulated cellular proliferation and thus contributes to the homeostasis of many tissues, but also provides an important safeguard against cancer. Thus, a further aspect of this invention is the use of the isolated human serum lectins of the present invention to specifically recognize abnormal or aberrant glycosylation present on cancer cells and effect their elimination through apoptotic processes.

Many cancer-specific carbohydrate epitopes have been well-characterized (Table 1). For example, the Tn antigen, representing N-acetylgalactosamine linked to the amino acids serine or threonine (GalNAc-R), is a tumour-associated antigen detectable by immunohistochemical techiques in most adenocarcinomas of the breast, colon, lung, bladder, endometrium and ovary (Itzkowitz S., et al., Gastroenterology, 100:1691-1700 (1991)). We have exploited this well-defined epitope to isolate proteins in human blood with specific affinity for this structure. Furthermore, we have shown that these serum proteins, operationally defined as lectins on the basis of carbohydrate-binding properties, potently cause apoptosis of cells expressing the cognate epitope, thus accounting for their highly selective anti-cancer properties. The apoptosis assay of the present invention is enabling technology with respect to both the identification and measurement of this biological property.

**TABLE 1**

**Cancer-associated antigens**

| Antigen | Structure |
|---|---|
| Tn | GalNAc alpha 2-Thr/Ser |
| Sialyl-Tn | Sialyl alpha (2,6) GalNAc alpha 2-Thr/Ser |
| T | Gal beta (1,3) GalNAc alpha 2-Thr/Ser (core 1) |
| Lewis$^x$ | Gal beta (1,4) (Fuc alpha (1,3)) GlcNAc- |
| Lewis$^y$ | Fuc alpha (1,2) Gal beta (1,4) (Fuc alpha (1,3)) GlcNAc- |
| Sialyl-Lewis$^x$ | Sialyl alpha (2,3) Gal beta (1,4) (Fuc alpha (1,3)) GlcNAc- |
| i | Gal beta (1,4) GlcNAc beta (1,3) Gal- |
| I | Gal beta (1,4) GlcNAc beta (1,6) (Gal beta (1,4) GlcNAc beta (1,3)) Gal- |
| mucin peptide | various |

We herein provide examples that endogenous serum proteins can recognize in a highly specific manner, bind, and cause apoptosis of malignant breast and lung cells expressing the immunodominant GalNAc (Tn antigen) structure. It is clearly anticipated that these serum proteins could be used to produce selective apoptosis of various cancer cells of diverse tissue origin which express this carbohydrate antigen. The method used for isolation of apogenic human serum proteins described in the present invention could readily be modified for the purposes of isolating functionally similar proteins which differ only in their antigen specificity. Of specific interest are the blood group-related carbohydrate structures designated as Le$^x$, sialyl-Le$^x$ and Le$^y$. The Le$^x$ antigens result from the synthesis of a fucose residue to N-acetyllactosamine, and the Le$^y$ antigen from the action of a specific fucosyltransferase on the blood group T antigen precursor, or H antigen. These terminal carbohydrate structures normally function as adhesion molecules, the increased expression of which has been strongly correlated with metastatic potential in a wide range of human tumours. For example, increased expression of sialyl-Le$^x$ is associated with a significantly worse prognosis in metastatic prostate cancer (Jorgensen T., et al., Cancer Research,

55: 1817-1823(1995)). Affinity chromatography incorporating these carbohydrate structures as ligands in order to bind and capture the corresponding proteins in human serum can be performed using substitution of these or other antigenic structures for the GalNAc moiety illustrated in the current examples. By analogy, such lectins would be anticipated to bind to these carbohydrate structures present in human cancers leading to the induction of programmed cell death. Additional mitigation of metastatic potential would bederived from interference with the adhesive function of the receptor in the case of $Le^x$ -, sialyl-$Le^x$-, and $Le^y$-antigen-bearing tumours.

The unifying theme of the present invention and not recognized in the prior art is a class of proteins present in human blood which have the capacity to selectively recognize and induce apoptosis of cells conveying abnormal surface carbohydrates. Alterations in cellular glycosylation can result from either species-specific differences or may reflect cancer-associated changes. Significantly, the proteins of the present invention exhibit minimal binding or reactivity against normally glycosylated human cells. These serum lectins are conceptually distinct from previously described mammalian apogens, such as TNF alpha, TGF beta, Il-8 and Fas ligand, which mediate their effects though high local concentrations. As an example TNF alpha mediates its effects by binding a receptor that triggers, through an as yet uncharacterized pathway, cellular apoptosis so that the distribution of the TNF alpha receptor determines the target cell specificity. This is also the case with the other well described biological apogens such as Fas ligand, TGF beta, and IL-8. The conceptual difference that distinguishes circulating lectins from the previously described apogens derives from the constitutive expression of the lectins, and this property provides surveillance for the detection and elimination of abnomally glycosylated cells. This is distinct from those cells expressing receptors specific for the lectins since, in the case of transformed cells, the expression of distinct carbohydrates represent a perturbation from normal glycosylation. In the case of breast cancer, the MUC-1 mucin expresses cryptic peptides and truncated carbohydrate chains consistent with the concept that normal glycosylation is readily

corrupted and that the glycan profile presents a specific signal recognized by circulating lectins.

Thus, the examples provided in this invention illustrate the utility of a conceptually new paradigm: that endogenous serum proteins recognize, in a specific manner, aberrant glycosylation present on the surface of cancer cells, thereby causing apoptotic elimination and serving as a serum-based (in contrast to classical cell-based immunity) anti-oncogenic surveillance mechanism.

Apoptosis Assay

The regulation of apoptosis is important in many pathological processes including xenograft rejection and cancer, and therefore there is a need for a method that can rapidly and quantitatively measure apoptosis. At present, most methods used to identify apoptosis rely on morphological assessment of cells or on biochemical criteria and are not suitable as high throughput screening assays. Many flow cytometry methods have been developed to quantify apoptosis (Fraker et al., Methods in Cell Biology, 46: 57-76 (1995); Ormerod et al., Acta Oncologica, 32: 417-24 (1993); Schmid et al., Cytometry, 15: 12-20 (1994); Schmid I., et al., Journal of Immunological Methods, 170:145-57 (1994).. Flow cytometry offers some advantages over assays such as DNA agarose gel electrophoresis and electron microscopy since it allows greater sampling capacity and may be less labor intensive. Nevertheless, several problems still remain: high cost, complex cell preparation, the need for large numbers of cells, and difficulties in determining gating. Thus, there is a need for a rpid inexpensive assay which uses non-radioactive methods to detect apoptosis. One aspect of the present invention is directed to such an assay.

In the present invention, it is shown that in the porcine-to-human model of xenotransplantation, most apoptotic cells lose their surface attachments and detach into the surrounding extracellular space. It was found that detachment was correlated with apoptosis, as detected by already established methods. Thus, the rate of

detachment can be used to quantify apoptosis in a fluorescent plate reader assay based on the uptake of supravital dyes by the remaining viable adherent cells. This invention provides a rapid, inexpensive, and simple assay for quantifying apoptotic porcine aortic endothelial cells (PAEC) cultured in 96-well plates using the fluorescent dye, Calcein AM. The rate of apoptosis determined with the detachment assay shows high correlation with that obtained by flow cytometric methods. We also show that the apoptosis of other cells such as cancer cells (T47D breast cancer cells, Calu-3 lung cancer cells), primary cells (human adipocytes), and normal human vascular cells (human pulmonary arterial, and human aortic endothelial cells) results in a quantitatively detectable degree of detachment which is measurable with the assay of the present invention.

Thus, one aspect of the present invention is directed to an apoptosis assay for the detection of apogens. The examples provided in this invention relate to the isolation of apogens or anti-apogens derived from combinatorial libraries, from phage display libraries of antibodies and peptides, from inorganic libraries, and from biological libraries such human serum. The apoptosis enables high throughput screening of these libraries for apogenically-active compounds based on the microtire plate format.

## SUMMARY OF THE INVENTION

The present invention relates to human serum apogens. Specifically, this invention relates to human serum lectins which induce apoptosis.

This invention also relates to a method of using said lectins to induce apoptosis in cells. Specifically, this invention relates to the capacity of said lectins to induce apoptosis in transformed and malignant cells.

This invention also relates to a method of blocking said lectins to inhibit apoptosis. More specifically, the present invention relates to a method of blocking said lectins to inhibit or reduce xenograft rejection.

This invention also relates to a novel bioassay for the detection and quantitation of cellular apoptosis *in vitro*.

This invention also relates to a method of inducing apoptosis comprising exposing cells to the human serum apogens of the present invention.

This invention also relates to a method of inducing apoptosis comprising exposing cells to the human serum lectins of the present invention.

This invention also relates to a method of inducing cytotoxicity comprising exposing cells to the human serum apogens of the present invention.

This invention also relates to a method of inducing cytotoxicity comprising exposing cells to the human serum lectins of the present invention.

According to the present invention, there is also provided a method of blocking cell death to thus inhibit xenograft rejection by blocking the action of the human serum lectins of the present invention. Interference of lectin-mediated apoptosis of xenogenic endothelial cells can be achieved using techniques including the production of monoclonal antibodies directed against apogenic lectins, specific immunoabsorption, and other techniques predicated on isolation of human serum lectin apogens.

This invention further relates to a method of isolating a human serum lectin that induces apoptosis. In essence, the method of isolating a human serum lectin capable of inducing apoptosis of porcine endothelial cells comprises the following steps:

a) applying human sera to a carbohydrate column to bind carbohydrate binding proteins;

b) eluting the bound proteins by washing the column with the respective

carbohydrate; and

c) collecting the isolated human serum lectin.

This invention further relates to a method of using said human serum lectins for the diagnosis and/or monitoring of cancer associated carbohydrate epitopes which may be either cell surface-bound or secreted.

This invention also relates to a novel bioassay for detecting and quantification of cellular apoptosis *in vitro*. Said method comprises the detection and quantification of cellular apoptosis using a fluorescence plate reader assay based on the uptake of supervital dyes by viable adherent cells.

This invention further relates to a method for the use of the apoptosis assay to screen large numbers of compounds for those with apogenic properties using the property of detachment as an indicator of apoptosis or cytotoxicity. These compounds could be selected from combinatorial libraries, from phage display libraries of antibodies and peptides, from inorganic libraries, and from biological libraries such human serum.

This invention further relates to the genes that encodes human serum lectins and the products of those genes as well as those genes that are sufficiently similar as to be in the same family of genes.

This invention further relates to compounds which are structurally similar to human serum lectins, especially the carbohydrate recognition domain, or compounds which are modified by substitution of amino acids which do not impair binding or apogenic capacity. These include compounds selected from combinatorial libraries, from phage display libraries of antibodies and peptides, from inorganic libraries, and from biological libraries such human serum.

This invention further relates to compounds which are structurally similar to human

serum apogens or compounds which are modified such that the modifications do not impair binding or apogenic capacity. These include compounds selected from combinatorial libraries, from phage display libraries of antibodies and peptides, from inorganic libraries, and from biological libraries such human serum.

This invention further relates to compounds which inhibit human serum lectin mediated apoptosis or cytotoxicity derived from combinatorial libraries, from phage display libraries of antibodies and peptides, from inorganic libraries such as carbohydrate libraries, from monoclonal antibodies or their fragments, and from biological libraries such human serum.

This invention further relates to the ligands of human serum lectins. According to the present invention, these ligands constitute epitopes present on xenogenic cells and human cells which express these epitopes.

This invention further relates to methods to modify cellular glycosylation to change the susceptiblity of cells to apoptosis mediated by human serum lectins or their analogues.

This invention also relates to a method of targeting drugs to an epitope of the present invention, wherein said method comprises physically linking said drug to a corresponding lectin of said epitope.

## BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings described below.

Figure 1 shows phase contrast microscopy (40x) of porcine endothelial cells treated with 25% human sera (Fig. 1a), 25% heat inactivated human sera (Fig. 1b), 25%

porcine sera (Fig. 1c), TNF alpha (40 ng/ml) (Fig. 1d), media only (Fig. 1e), and 10% fetal calf serum (Fig. 1f) for 18 h. Apoptotic cells appear as rounded clear cells compared to normal cells with cobblestone morphology.

Figure 2 shows porcine endothelial cells treated with 25% human serum for 6 h, 12 h, 18 h, and 24 h (Fig. 2a, lanes 1-4), 25% porcine sera, 25% heat inactivated human sera, media only all for 24 h (Fig. 2b, lanes 1-3), and freeze/thawing (Fig. 2b, lane 4). Included are 100 bp markers on the 1.8% agarose gel stained with ethidium bromide. DNA ladders indicative of apoptosis were seen in cells treated with human sera, but not in porcine endothelial cells treated with porcine sera, heat inactivated human sera, media, or freeze/thawing, which is indicative of necrosis but not of apoptosis.

Figure 3 depicts terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) of detached cells 24 h following treatment with human sera (Fig. 3a), heat-inactivated human serum (HIHS) (Fig. 3b), TNF alpha (Fig. 3c), or media (Fig. 3d); and detached cells from cells treated with human sera (Fig. 3e) for 24 h or freeze/thawing (Fig. 3f). Digoxigenin-labelled dUTP was detected by FITC anti-digoxigenin and fluorescence microscopy (100x). Propidium iodide was used for counter staining. Note the presence of very few apoptotic cells in freeze/thawed cells (arrows).

Figure 4 shows adherent (Fig. 4a) and detached (Fig. 4c) porcine endothelial cells treated with 25% human sera for 24 h and stained with Hoechst-33258 and propidium iodide prior to fixation and examined by direct fluorescence microscopy (100x). TNF alpha-treated porcine endothelial cells (Fig. 4b, 40 ng/ml for 16 h) as a positive control, and cells treated by freeze/thawing (Fig. 4d) as a negative control, were similarly stained. Note apoptotic cells with apoptotic bodies and nuclear condensation (arrowheads) and necrotic cells (arrows).

Figure 5 shows the electron microphotographs of porcine endothelial cells treated with 25% human sera for 24 h (10,000x). During the initial induction of apoptosis

there is condensation and margination of nuclear DNA (Fig. 5a, arrowhead). As apoptosis proceeds, there is a decrease in cell volume as the cells lose their surface attachments and become round in shape; there is also endoplasmic reticulum dilation and cell membrane ruffling (Fig 5b, arrow). In later stages of apoptosis (Fig. 5c) there is marked condensation of DNA (arrowhead), and formation of apoptotic bodies (long arrow), in contrast to a normal cell (short arrow). During resolution of apoptosis, adjacent cells sometimes phagocytose apoptotic cells (Fig. 5d, arrow).

Figure 6 shows the DNA histograms of cells treated with 25% human sera for 18 h (Fig. 6a), TNF alpha (40 ng/ml) for 16 h (Fig. 6b), or with 25% porcine sera for 18 h (Fig. 6c). Cells were stained with propidium iodide and analyzed by fluorescence activated cell sorting (FACS). A graph of hypodiploid fractions of cells treated with 25% human serum and 25% porcine serum over time is shown in Fig. 6d.

Figure 7 shows cells treated with 25% heat-inactivated human sera for 1 h (Fig. 7a) or 24 h (Fig. 7b) and cells treated with 25% heat-inactivated human sera with supplementary guinea pig sera as a source of complement for 1 h (Fig. 7c) or 24 h (Fig. 7d) were stained with Calcein AM/ethidium homodimer . Cells treated with 25% heat-inactivated human sera with supplementary guinea pig sera for 1 h (Fig. 7e) or 24 h (Fig. 7f) were stained with Hoechst-33258 and propidium iodide. Note that the number of apoptotic cells with condensed nuclei and apoptotic bodies (some of which are indicated by arrow heads) were not increased with addition of exogenous complement (e and f), whereas the number of necrotic cells (some of which are indicated by arrows) decreased with time (c and d).

Figure 8 shows the SDS-PAGE of human alpha galactosyl binding proteins. Carbohydrate binding proteins from human sera were purified on a p-aminophenyl-(alpha)-D-galactose (papGal) carbohydrate column, then separated on a DEAE column, resolved by reducing SDS-PAGE, and stained with Coomassie Blue. Human IgM and IgG are included for reference (lanes 1 and 2, respectively). Four fractions from the DEAE column are shown (lanes 3-6), as well as the isolates

from the papGal column (lane 7). Note the single band of 73 kD molecular weight in lane 5. Molecular weight standards are indicated by arrows.

Figure 9 shows porcine endothelial cell apoptosis induced by isolated human carbohydrate binding proteins from Fig. 8, lane 7. Porcine endothelial cells were incubated for 18 h with papGal carbohydrate binding proteins isolated from human sera. The detached (Fig. 9a) and adherent cells (Fig. 9b) were stained with Hoechst-33258 and propidium iodide and examined by direct fluorescence microscopy (long arrows indicate apoptotic bodies). Note nuclear condensation (arrowheads), shrinkage of apoptotic cells, and lack of staining by propidium iodide. The detached (Fig. 9c) and adherent cells (Fig. 9d) were also examined with Apoptag staining (TUNEL assay), to determine endonuclease activity indicative of apoptotic cells. Apoptotic cells are brightly fluorescent.

Figure 10 demonstrates the loss of porcine endothelial cell surface attachments during apoptosis. Porcine endothelial cells incubated with papGal-binding proteins for 18 h at the noted initial concentration (in parenthesis in legend) underwent dose-dependent detachment, as determined by the number of adherent cells which were able to metabolize Calcein AM. The amount of detachment was compared to cells incubated with media alone. The single band isolate, papGal 73, from DEAE chromatography produced a greater degree of detachment, as an indication of apoptosis, compared to that of human IgM and TNF alpha.

Figure 11 demonstrates that plant lectins can produce porcine endothelial cell detachment indicative of apoptosis. Some plant lectins (initial concentrations in parenthesis in legend) produced dose-dependent porcine endothelial cell detachment after 18 h. BSI4, wheat germ agglutinin (WGA), and soybean agglutinin (SBA) produced 70-80% maximal detachment. PNA, TPP, and MPA did not produce porcine endothelial cell detachment.

Figure 12 shows the fluorescence microscopy of apoptotic endothelial cells.

Fluorescence microscopy of PAEC treated with 25% human serum for 18 h. Adherent cells (Fig. 12a) and detached cells (Fig. 12b) were stained with Hoechst 33258 dye. Most of detached cells showed apoptotic morphology such as condensed chromatin, apoptotic bodies, or nuclear blebbing. Adherent cells (Fig. 12c) and detached cells (Fig. 12d) were labelled by the TUNEL method. Almost all detached cells were positively stained, whereas very few adherent cells were positively stained (400X).

Figure 13 demonstrates that human serum (HS) and TNF alpha produce dose-dependent PAEC apoptosis. PAEC were treated with increasing concentrations of TNF alpha (Fig. 13a) or HS (Fig. 13b) for 18 h. FACS analysis was performed on the PAEC after TUNEL staining or propidium iodide staining. Cell detachment was quantified by a fluorescent plate reader assay based on the uptake of Calcein AM by adherent cells. The regression equations and correlation coefficients are depicted in the figure. The regression lines for each of the three assays were significant by ANOVA ($p<0.05$), with the exception of the FACS-DNA data in the case of TNF alpha ($P=0.09$).

Figure 14 shows the correlation of the detachment assay with the TUNEL assay. There was a high degree of correlation between percentage detachment and percentage PAEC apoptosis detected by TUNEL after 18 h incubation with HS or TNF alpha ( Triangle-HS (10, 20, 30, 40, and 50%); Square-TNF alpha (8, 16, 24, 32, and 40 ng/ml)). The lines shown were fitted by linear regression analysis. $P=0.04$ for HS treated cells, and $P=0.01$ for TNF alpha treated cells by ANOVA. The slopes and intercepts of the lines were not different ($p=0.81$ and $p=0.90$ respectively by ANCOVA).

Figure 15 demonstrates that DS-OSM binding lectins can produce T47D detachment indicative of apoptosis. Human serum lectins isolated by affinity chromatography using DS-OSM, pap-Gal and melibiose (300 µg/ml initial concentration) were incubated with T47D cells for 24 h. The columns were also eluted without prior

application of human serum, and the eluants (GalNAc and galactose buffer) as well as cyclophosamide (CPA), human sera and PBS were tested in the assay. The percentage detachment was detected in a fluorescent plate reader assay and depicted relative to detachment produced by PBS. DS-OSM binding lectins produced similar levels of detachment as the positive control (CPA which produces non-specific toxicity), whereas the other serum lectins did not have such an effect.

Figure 16 shows that DS-OSM binding lectins produce detachment of Calu-3 cells indicative of apoptosis. Calu-3 cells were treated with DS-OSM binding lectins (30 µg/mL), CPA (10 mg/mL), LBA and DBA (both at 300 µg/mL) for 24 h. The percentage detachment was detected in a fluorescent plate reader assay and depicted relative to detachment produced by PBS. DS-OSM-binding lectins produced similar levels of detachment as the positive control (CPA), whereas the plant lectins did not have such an effect.

Figure 17 shows the carbohydrate specificity of SBA-induced detachment. SBA plant lectin produces detachment of PAEC (diamonds), but the monosaccharide GalNAc did not produce detachment (squares) of PAEC. When SBA was used to induce PAEC apoptosis GalNAc was able to completely inhibit the pro-apoptotic effects (triangles) caused by 180 µg/ml of SBA.

## DESCRIPTION OF PREFERRED EMBODIMENT

The present invention relates to human serum proteins shown to cause apoptosis of cancer cells and xenogenic endothelial cells. This invention also relates to a method using said lectins to induce apoptosis in transformed or malignant cells. This invention also relates to methods to achieve blocking of said lectins to inhibit apoptosis associated with xenograft rejection. This invention also relates to a method of isolating said lectins. This invention also relates to both xenogenic and cancer-related epitopes which mediate apoptosis via interaction with human serum lectins. This invention also relates to a novel bioassay for the detection

and quantification of cellular apoptosis *in vitro*, and to the utility of this assay in measuring the apogenic properties of biological and synthetic compounds. In this invention the abbreviations shown in the following Table have been used.

**Table of abbreviations**

| | |
|---|---|
| Ab | Antibody |
| bp | Base pairs |
| Calcein AM or CAM | Calcein acetoxymethyl ester |
| ConA | Concanavalin A |
| FACS | Fluorescence activated cell sorting |
| FACS-DNA | Fluorescence activated cell sorting-Deoxyribonucleic acid content analysis |
| FITC | Fluoroisothiocyanate |
| HS | Human serum/sera |
| LCA | Lens culinaris agglutinin |
| PAEC | Porcine aortic endothelial cells |
| PI | Propidium iodide |
| PHA | Phytohaemagglutinin |
| TNF alpha | Tumour necrosis factor alpha |
| TUNEL | TdT(terminal deoxynucleotidyl transferase)-mediated dUTP-biotin(ordigoxigenin) nick-end labelling |
| EHD | Ethidium homodimer |
| HIHS | Heat inactivated human serum/sera |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| papGal | p-Aminophenyl-(alpha)-D-Galactose |
| GalNAc | N-acetyl galactose |
| GlcNAc | N-acetyl glucose |
| DS-OSM | Desialyated ovine submaxillary mucin |
| GT | Glycosyl-transferase |
| MBP | Mannan-binding protein |
| WGA | Wheat germ agglutinin |

Human serum lectins and xenograft rejection

Xenotransplantation between discordant species, such as in porcine-to-primate combinations, is complicated by xenograft rejection. Although preformed antibodies

and complement may participate in the pathogenesis of xenograft rejection, we herein provide evidence of a novel mechanism which targets the vascular endothelium and which may contribute to xenograft rejection. Characterization of the apoptotic pathway occurring during xenograft rejection has important implications, since apoptosis is a regulated process and may be amenable to manipulation by molecular biology techniques. Delineation of a novel pathway of apoptosis in this context may provide further general understanding of apoptotic mechanisms. Finally, identification of human apogens will produce more specific therapy for treatment of discordant xenograft rejection. According to the present invention, it has been demonstrated that human serum proteins, including lectins and antibodies, mediate porcine endothelial apoptosis through carbohydrate xenogenic epitopes.

The term "apoptosis" is intended to describe a cellular process of cell death characterized by the presence of cell shrinkage, nuclear collapse, and a characteristic pattern of DNA fragmentation.

The term "lectin" was originally defined as a multivalent, non-immunoglobulin, carbohydrate binding molecule, operationally defined by its ability to agglutinate cells bearing appropriate saccharide structures. More recently, the term has been used to include proteins with a single carbohydrate binding site.

According to the present invention and as illustrated in Figures 1-14, porcine endothelial cells treated with human serum undergo detachment from the culture plates and exhibit morphological features of apoptosis such as shrinkage, membrane blebbing, nuclear condensation, and formation of apoptotic bodies. Additionally, biochemical evidence of apoptosis of the cells was revealed, including DNA laddering on agarose gels and positive staining at the single cell level with the TUNEL method. Further, there was ultrastructural evidence of apoptosis, nuclear condensation, DNA clumping, margination, nuclear clefting, and endoplasmic dilation in association with intact cellular membranes. Noteworthy were isolated examples of cells undergoing phagocytosis by neighbouring cells, a phenomenon associated

with apopotic, but not necrotic, cell death.

The induction of porcine endothelial cell apoptosis in this xenogenic model is likely due to a heat-labile humanapogen resulting from a positive stimulus, since the porcine endothelial cells were resistant to apoptosis using heat-inactivated human sera. The extent of apoptotic injury resulting from the addition of exogenous complement did not approach that observed with human serum and would suggest that complement-mediated membrane damage is not sufficient by itself to induce apoptosis. We also show that papGal-binding lectins and DS-OSM-binding lectins isolated from human serum causes a dose-dependent apoptotic effect in PAEC, and that in some cases this effect is mimicked by exposure to plant lectins having the same specificity.

Xenogenic Epitopes

Strategies designed to defeat hyperacute xenograft rejection have focused on the singular importance of the carbohydrate epitope, Gal alpha (1,3) Gal (Vaughan H. A., Transplantation, 58:879-882(1994); Good A.H., et al., Transplant Proc, 24:559(1992)). We have identified herein the existence of a new protein present in human serum which exhibits specific affinity for this epitope expressed on porcine endothelial cells and, which upon binding to this epitope, directly leads to apoptosis of the cell conveying the epitope (Example 2). Further to this discovery, we show that a funtionally analogous but structurally distinct protein can bind to a different carbohydrate epitope, the GalNAc (Tn antigen) structure (Example 5), and cause apoptosis in a manner highly similar to that of the Gal alpha (1,3) Gal epitope lectin. Thus, the discovery of human serum lectins leads to the definition of additional xenogenic epitopes as exemplified by the case of GalNAc recognized by DS-OSM binding lectins.

Prevention of Xenograft Rejection

According to the present invention, there is also provided a method of inhibiting xenograft rejection by blocking the action of human serum apogens or human serum lectins which induce apoptosis. Compounds that block human serum lectins or human serum apogenic activity can be derived from the following: combinatorial libraries, synthetic peptide libraries and monoclonal antibodies (mAb) or fragments derived from monoclonal antibodies. These could be used as competitive lectin inhibitors to bind the lectin or the same receptors as the authentic lectin in a way that does not induce apoptosis and thereby serving a blocking function to attenuate xenograft rejection. The function and efficacy of competitive lectin blockers could be evaluated using screening methods, such as the apoptosis assay of the present invention, in order to detect a reduction in the rate of apoptosis. Other methods which could be used to attenuate xenograft rejection include: the use of carbohydrate analogues which mimic the endogenous carbohydrate ligand and thus competitively antagonize lectin binding; the use of anti-lectin monoclonal antibodies to tie up the lectin binding site in solution, to modulate or down-regulate the production of lectins, or to delete the cells that produce the lectin (described in Example 7); the use of monoclonal antibodies directed against xenogenic antibodies which could inhibit said lectins by virtue of expected cross reactivity with antigen binding sites present on both the lectins and cognate antibodies could also be used to attenuate xenograft rejection.

(a) Anti-rejection strategies

The reagents which block lectin binding including peptide mimetics of the lectin ligands, carbohydrate analogues, anti-lectin antibodies, anti-antibody antibodies, and glycoprotein analogues, could be infused intravenously or immobilized on a solid matrix to deplete the lectins from blood. Another method to decrease lectin activity is the perfusion of vascularized organs from the donor animal in order to deplete the relevant lectins by deposition on the xenogenic endothelium.

Drugs which affect the level of endogenous serum levels could also be used to treat the xenograft recipient prior to transplantation. Methods provided in the present invention to test for lectin activity using the apoptosis assay will enable the screening for drugs which affect lectin levels.

In addition to strategies to treat the recipients of xenograft organs or cells, strategies to treat the donors can be logically derived based on the knowlege that human serum lectins produces apoptosis of xenogenic cells. We have produced Bcl-2 transfected endothelial cell lines that are resistant to TNF alpha, a common apogen, and may therefore be resistant to human serum lectins of the present invention. The donor pig could be somatically transfected prior to transplantation with Bcl-2 or other genes which protect against apoptosis, such as A1, which is the Bcl-2 analogue present in endothelial cells. These genes could also be used to make transgenic donor pigs which may be resistant to apoptosis mediated by human serum lectins. Other ways of altering the susceptibility of porcine cells to apoptosis include the use of glycosyl-transferase (GT) inhibitors to alter glycosylation of the endothelial cells in order to evade lectin binding. These glycosyl-transferase inhibitors could be administered orally or intravenously to pigs.

Breeding strains of pigs which are less susceptible to endothelial cell apoptosis could be employed to reduce xenograft rejection, the success of this approach being dependent on a determination of the effect of isolated human serum lectins on genetically diverse porcine tissues.

(b) Isolation of Apogenic Proteins

(i) Gal alpha lectin:

In order to isolate human serum apogens that cause porcine endothelial cell apoptosis, human carbohydrate-binding proteins were isolated on several different carbohydrate columns. In an example of the present invention, the carbohydrate columns were

selected from Gal alpha (papGal, and melibiose), GlcNA, fucose, and mannose sugars as well as desialyated ovine submaxillary mucin (containing predominately GalNAc sugars). The lectins of the present invention were the corresponding structures that bound to these columns. Other carbohydrate columns, such as the N-(e-aminocaproyl)-beta-D-galactopyranosylamine, N,N'-diacetylchitobiose, GalNAc columns (only GalNAc linked to resin) or the resin control did not bind serum proteins which exhibited the property of inducing apoptosis.

The isolates from Gal alpha columns were further fractionated using ion exchange chromatography, specifically, DEAE chromatography, which produced four fractions. The molecular weights of the major proteins purified by DEAE chromatography were 73 kD, 60 kD, 32 kD, and 33 kD.

The 73 kD protein eluted using 200 mM NaCl was a single band on reducing SDS-PAGE. These papGal binding proteins produced porcine endothelial cell detachment indicative of porcine endothelial cell apoptosis. Human serum lectin proteins from the GlcNAc, fucose, mannose, lactose, and melibiose columns also produced apoptosis. Similar concentrations purified IgM or IgG isolated from Protein G columns produced significantly less apoptosis of xenogenic cells, notwithstanding the putative importance of the anti-Gal-alpha-3Gal antibody in promoting xenograft rejection.

(ii) GalNAc lectin:

In an another example of the present invention, fractions eluted from a DS-OSM column with GalNAc were concentrated and subjected to polyacrylamide gel electrophoresis under reducing and native gel conditions with Coomassie Blue staining. Under reducing conditions there were seven major bands corresponding to the following proteins by amino acid sequencing: 45kD-alpha 1 antitrypsin, 70kD-albumin, 100kD-alpha macroglobulin, 80kD-IgG heavy chain and transferrrin,

30kD-IgG light chain, 60kD-IgG heavy chain, 40kD-haptoglobulin and an unknown protein with a partial amino acid sequence: I/LKSDALKSVSQGP. The eluant from the DS-OSM column is shown in Example 4 to be a potent apogenic stimulus in breast and lung cancer cells and, in distinction to the cancericidal agent, cyclophosphamide, to produce little or no effect on normal control cells. Separate evaluation of albumin, transferrin, alpha 1 antitrypsin, alpha macroglobulin, human antibodies including the anti-sialyl-Tn antibody, all of which exhibited no apogenic effect in isolation,indicates that the apogenic effect of the DS-OSM binding lectin is attributable to the unknown protein (alone or in combination with the other DS-OSM-binding proteins) containing the partial amino acid sequence given above.

Thus, suitable methods for the isolation of human serum lectins responsible for apoptosis can include affinity column purification using suitable carbohydrate ligands, followed by additional chromatography to provide further separation of the human lectins. For example, DEAE column chromatography has been found to be useful to separate different fractions of human lectins isolated on carbohydrate columns.

## LECTINS MEDIATE CANCER CELL DEATH

### Altered glycosylation in cancer

Oncogenic transformation in many tissues is associated with altered glycosylation or the appearance of oncofetal glycoforms. For example, the development of colon cancer occurs in several steps which include the activation of oncogenes and inactivation of suppressor genes. Glycosylation changes arising during carcinogenesis most likely originate as a consequence of malignant transformation and changing growth and differentiation of the cancer cell. These glycosylation changes are critical for the biology of the cancer cell and influence their immunogenicity, cell adhesion

and other properties. A mutated gene found in colon cancer appears to code for a member of the adhesion molecule family, supporting the idea that extracellular interactions are important in the control of cell growth. The regulation of glycosyltransferase gene expression may be linked to the activation of oncogenes. An important challenge is to understand the control of glycosylation during malignant transformation (Brockhausen I. and Kuhns W. Glycoproteins and human disease. Medical Intelligence Unit, London Pub Ed. Chapman S. and Hall (1997)).

Analysis of the detailed structures of glycopeptides and oligosaccharide chains has been useful in evaluating discrete alterations present in tumours. In many cases, cancer associated carbohydrate alterations include increased expression of truncated forms of O-glycans, increased branching of complex N-glycans associated with increased poly-N-acetyllactosamines and expression of GlcNAc-transferase V, and altered sialylation, sulfation and fucosylation and other types of glycosylation. These changes may be a result of altered expression of glycosyltransferases and sulfotransferases. Alternatively, peptide moieties of glycoproteins may change in cancer and lead to increased peptide epitope expression as well as changes in peptide-specific glycosylation.

Tissues and sera from lung cancer patients contain sialylated and fucosylated poly N-acetyl-lactosamine type 1 and type 2 chain epitopes are regarded as general carcinoma antigens. These epitopes have been found on oncofetal types of glycoproteins and glycolipids. In one embodiment of the present invention, human serum lectins which bind to desialyated ovine submaxillary mucin show cytotxic activity against a lung adenocarcinoma cell line.

Human serum lectins which induce apoptosis

According to one aspect of the present invention, human serum lectins with affinity to Tn antigen, an immunodominant breast and lung cancer epitope, were isolated by

affinity chromatography using desialyated ovine submaxillary mucin. Several DS-OSM-binding protein fractions have been identified which have been shown to produce apoptosis of human breast and lung cancer cells *in vitro* in a potent and highly specific manner. The present invention indicates that highly effective killing of breast cancer cells and lung cancer cells occurs on exposure to small amounts of isolated human serum lectins, whereas apoptotic injury to human preadipocytes, human pulmonary arterial cells, and human aortic endothelial cells is conspicuously absent. The vascular endothelium is a limiting site of toxicity of intravenous routes of administration. This implies a favourable efficacy/toxicity ratio which, if substantiated *in vivo*, would be superior to that of chemotherapeutic regimens in current clinical use. Not wanting to be bound to any particular theory, it is suggested that the human serum lectins of the present invention represent a primitive, and therefore highly conserved, class of human serum proteins, and as such, the administration of these lectins is unlikely to provoke serious allogeneic effects which would mitigate their *in vivo* biological activity.

Human serum lectins as cytotoxic agents

The human apogen or human lectins of the present invention can be used as a cytotoxin to induce apoptosis in tumour cells. The human serum lectins of the present invention comprise an innate anti-cancer host defense mechanism. The lectins of the present invention show specific recognition of anomalous or incomplete glycosylation of cancer cell surfaces. The human serum lectins of the present invention can thus be used for the development of novel cancer therapies.

Thus, according to the present invention there is provided a new paradigm of tumour biology based on the interaction of tumour cell surface glycoconjugates and endogenous lectins. Although not bound by any particular theory, it is suggested that endogenous lectins, which are carbohydrate-binding molecules but distinct from immunoglobulins, recognize in a specific manner aberrant glycosylation of mucins present on the surface of nascent breast cancer cells, thereby causing

apoptotic signalling and elimination of cancer cells.

The finding that human lectins induce apoptosis in cancer cells represents a new insight regarding endogenous pathways for suppression of oncogenesis and provides the basis for conceptually new immunotherapeutic approaches to cancer.

There is precedent for the use of plant lectins for inducing cancer cell death, and there is one report of apoptosis in cancer cells induced with a plant lectin (Janssen O., et al., Arzneimittel Forschung Drug Research, 43 (11): 1221 (1993); Kim M., et al., Glycobiology, 3 (5): 447(1993); Chen Y.F., et al., International Journal of Cancer, 57 (4): 561 (1994)). The human lectins of the present invention appear to operate by the same mechanism as certain plant lectins, that is, by ligation of a similar carbohydrate epitope which then initiates the apoptotic cascade.

The logic of using the described human serum lectins to target cancerous cells is based on the similarity in glycoprotein antigens present on xenogenic (porcine) endothelial cells and those expressed in many cancer cell lines, specifically GalNAc structures, and based on the observation that plant lectins with corresponding specificities exhibit cytotoxicity towards specific cancers. The capacity of lectins to specifically bind to cancer specific epitopes can be exploited by a combination of said lectins with cytotoxic radiopharmaceutical agents or other cytotoxic agents. The ligands defined by these lectins would thus identify new apoptosis-transducing epitopes and define new functions for previously described epitopes. The human apogens or human lectins of the present invention can be used as a cytotoxins to induce apoptosis in tumour cells. The lectins of the invention, when combined with a pharmaceutically acceptable carrier, can be used in compositions suitable for pharmaceutical administration. The examples of the present invention are limited to breast cancer cells and lung cancer cells; however, the invention itself is not so limited. The human lectins of the present invention are capable of inducing apoptosis in a wide range of transformed or malignant cell type based on their expression of the cognate ligand such as GalNAc.

Derivative strategies

The information obtained from amino acid sequencing of lectins presented in this invention and from further lectin sequences could be used to clone out the genes for the lectins and expressed. Combinatorial libraries derived from these genes could be constructed and displayed in phage display systems for the isolation of more potent variants of the lectins. The primary amino acid sequence could also be used to construct variant peptides using protein synthesis chemistry to produce more potent analogues of the lectins. One potential approach to increase the potency would be to synthesize multivalent proteins of lectin or lectin analogues. These techniques are commercially available and widely used. These lectin variants, as well as the isolated lectins, could be administered intravenously to target cancer cells to produce apoptosis. Alternatively, the lectins may be encapsulated in a lipid coat for absorption via oral administration.

The biological activity of isolated human serum lectins could be evaluated based on their capacity to bind and induce apoptosis of various cancer cell lines as well as human tumour specimens. Binding of human lectins to cancer cells could be ascertained by immunofluorescence using monoclonal antibodies to the human lectins or by radioiodination of the purified lectins.

These experiments would be logically guided by concurrent studies of tumour-specific glycosylation to determine the relevant lectin specificities. *In vivo* experiments to corroborate these findings using a tumour-bearing SCID mouse model are within the scope of the present invention.

The development of novel glycosyltransferase modifiers based on the analysis of cancer-specific GT activity would be anticipated to yield compounds which affect the susceptibility of treated cells to apoptotic injury caused by human serum lectins. Modulation of surface-bound glycoconjugates unique to cancer cells by GT inhibitors could be used to interfere with their susceptibility to lectin-mediated apoptosis.

A method for detecting cellular apoptosis

According to the present invention there is also provided a method of detecting cellular apoptosis. Endothelial cells undergo a number of morphological and biochemical changes during apoptosis. Porcine endothelial cells cultured with TNF alpha, a known apogen, or with human serum acquire a spherical shape, in comparison to their normal flat cobblestone shape, and show surface blebbing when observed by inverted phase constrast microscopy.

Examples of suitable metabolic live dyes include: Calcein AM, Carboxycalcein, Fluorescein diacetate, BCECF, and Carboxynaphthofluorescein diacetate. Generally, the method involves the addition of the test apogens to cells grown to confluency in microtitre plates. After a test period has elapsed, the detached apoptotic cells are removed from the plate by washing, and the metabolic live dye is loaded into the adherent live cells and subsequently quantified in a fluorescent plate reader.

While this invention is described in detail with particular reference to preferred embodiments thereof, said embodiments are offered to illustrate, but not limit, the invention.

EXAMPLES

Example 1: Induction Of Porcine Endothelial Cell Apoptosis By Human Serum

Porcine aortic endothelial cells were isolated from aortas obtained from an abattoir. The cells were cultured in 10% fetal calf serum (Immunocorp, Montreal, QE) in M199 cell culture media and passaged using Trypsin-EDTA (all cell culture reagents were obtained from Gibco BRL, Grand Island, NY, unless otherwise indicated) in 100 mm culture dishes (Falcon, Oxnard, CA). Cell type was confirmed by uptake of acetylated LDL and by cobblestone morphology on phase contrast microscopy. Cells from passages 4-10 were passaged into appropriate sized culture plates (e.g. 96 well

plates, or chamber slides (Nunc, Naperville, IL)) and allowed to come to confluency over 4 days prior to experiments. All human AB serum (from hospital blood bank) and all pig sera (collected from the abattoir) were diluted to 25% (unless otherwise specified) with M199 (all reagents were diluted with M199 unless specified). Pools of human and pig sera consisted of at least three donors. Although AB sera was used in this example, all other blood types are equally useful for this purpose.

On the day of the experiment, the old medium was removed and the cells were washed with PBS (Gibco BRL) twice. Either human sera, human TNF alpha (Calbiochem, La Jolla, CA) at 40 ng/ml (reconstituted from a 1 mg/ml stock solution), or pig sera were incubated with porcine endothelial cell cultures. In experiments to determine time dependency of apoptosis, the cells were incubated for variable times indicated in the figures; otherwise, there was a 24 h incubation period for human and pig sera, and a 16 h incubation period for TNF alpha.

Porcine endothelial cells were incubated for 18 h with TNF alpha, M199, pig sera, and human sera, and photographed with a Nikon microscope with phase contrast filters and a Nikon 601M camera with Kodak Ectachrome slide film. All slides were digitized using the Nikon Scanner LS-10E and a Apple MacIntosh Quadra 840AV computer.

Porcine endothelial cells were passaged into chamber slides (Nunc) and grown for 4 days. On the day of the experiment, the porcine endothelial cells were washed with PBS and incubated with either human sera, pig sera, heat-inactivated human sera, or heat-inactivated human sera supplemented with 20% guinea pig sera (Seralab, Sussex, England). At 1 h and 24 h, the membrane permeability and nuclear morphology of the treated cells were examined by fluorescence microscopy. To assess membrane permeability, the porcine endothelial cells were stained with Calcein AM (CAM) (retained by cells with intact membranes), and ethidium homodimer (EHD) (excluded by cells with intact membranes) (Molecular Probes, Eugene, OR). These dyes are

used to detect cells which are necrotic but not apoptotic, since necrotic cells do not preserve their membrane integrity, whereas apoptotic cells do. CAM and EHD stock solutions were stored separately at -20°C at 1 mg/ml in DMSO (Fisher Scientific, Fairlawn, NJ). The CAM-EHD working solution was made immediately prior to use by combining 4 μM CAM and 6 μM EHD in M199. To assess nuclear morphology the cells were stained with propidium iodide (PI) and Hoechst 33258 (Ho258). PI and Ho258 stock solutions were stored at 4°C at 10 mg/ml in sterilized water. The PI-Ho258 working solution was made immediately prior to use, by combining 50 μg/ml PI and 100 μg/ml Ho258 in M199. Staining was carried out by removing the sample solutions and adding the 500 μl of the above dyes for 30 min at 37°C. The dye was aspirated and the cells were washed once with PBS, and fixed with 0.5% paraformaldehyde for 30 min at room temperature. The fixed cells were then washed twice with PBS and examined with a fluorescence microscope (Olympus, Carsen Medical, Markham, ON). Two different emission filters were used: 420 nm (for the CAM-EHD) and 570 nm (for the PI-Ho258). The membrane damaged cells were quantified by enumerating the cells that showed uptake of EHD and release of CAM. The apoptotic cells were quantified by enumerating the cells that demonstrated features of apoptosis, specifically: nuclear condensation, decreased cell volume, and formation of apoptotic bodies. Triplicate experiments with greater than 500 cells were counted for each experiment.

Porcine endothelial cells cultured with human sera randomly detached from the tissue culture plates (Fig. 1a) in a time dependent fashion associated with acquisition of a rounded cell shape, blebbing, and cell shrinkage. Porcine endothelial cells treated with TNF alpha demonstrated accelerated cell detachment as compared to porcine endothelial cells treated with human sera (Fig. 1d). TNF alpha is known to produce apoptosis in endothelial cells (Polunovsky V.A., et al., Experimental Cell Research, 214(2): 584-94 (1994)). The appearance of porcine endothelial cells treated with human sera resembled the appearance of cells treated with TNF alpha, in that the TNF alpha treated cells also exhibiting rounding-up, blebbing, and decreased size. In contrast, porcine endothelial cells treated with either porcine sera, heat-inactivated

human sera, media alone, or 10% FCS showed very low cell detachment rates (Fig. 1b, 1c, 1e, and 1f).

To determine whether cells treated with human serum were undergoing apoptosis, these cells were examined for morphological and biochemical evidence of apoptosis. The TUNEL assay was performed on adherent and detached porcine endothelial cells treated with human sera, or treated with TNF alpha using the Apotag kit according to manufacturer's instructions (Oncor, Gaithersberg, MO). Isolation of DNA for agarose gel electrophoresis was performed according to the method of Herrmann with some modifications (Herrmann M., et al., Nucleic Acids Research, 22(24): 5506-7 (1994)). In brief, after treatment with human sera or pig sera, the adherent cells were washed with PBS and added to detached cells to be pelleted with detached cells at 500 xg for 10 min at room temperature. The adherent cells and pelleted cells were treated with 500 µl of 1% NP-40/20 mM EDTA/50 mM Tris HCL, pH 7.5, each for 1 min (Sigma, St. Louis, MO), then added together for centrifugation at 1,600 xg for 10 min at 4°C. The supernatant was decanted and treated with 8 µl of 12 µg/ml of RNAase for 2 h at 56°C and then 5 µl of 20 µg/ml of proteinase A (Pharmacia, Uppsala, Sweden) for 2 h at 56°C. The DNA was precipitated with 100 µl of 5M NaCl and 700 µl of 70% isopropanol (Fisher Scientific) overnight at -20°C. The DNA was pelleted by centrifugation at 21 000 xg for 30 min at 4°C and then solubilized in Tris-EDTA and analyzed on 2% (w:v) agarose gel with equal volume loading. Endonuclease activation and internucleosomal DNA cleavage producing DNA ladders of 180-200 bp repeats on agarose gels has been described as a hallmark of apoptosis in many systems (Arends M.J., et al., American Journal of Pathology, 136(3): 593-608 (1990)). Human sera treated porcine endothelial cells produced DNA ladders after 18 h (Fig. 2a, lanes 1-4), but those treated with porcine sera, heat-inactivated human sera, or media, did not (Fig. 2b, lanes 1-3). Cells killed by necrosis induced by freeze/thawing produced a smear on agarose gel, and not a DNA ladder (Fig. 2b, lane 4) indicative of random digestion.

DNA cleavage at the single cell level could be detected by TUNEL (Gavrieli Y., et al., Journal of Cell Biology, 119(3): 493-501 (1992)). Cells treated with human serum for 24 h were positively labelled whereas those treated with porcine sera, heat-inactivated human sera, or media had minimal labelling. Treatment of porcine endothelial cells with human sera lead to detachment of cells. Greater than 90% of these detached cells showed labelling (Fig. 3a); in contrast, detached cells produced by freeze/thawing had minimal labelling by the TUNEL assay (Fig. 3f).

Because endonuclease activation is not pathognomonic of apoptosis, morphological and ultrastructural studies are required to demonstrate an apoptotic mechanism of cell death (Cohen G.M., et al., Biochemical Journal, 286 (Pt 2): 331-4 (1992); Collins R.J., et al., International Journal of Radiation Biology, 61(4): 451-3 (1992)). Human sera treated porcine endothelial cells with the freely permeable DNA binding fluorescent dye Ho258 revealed randomly distributed cells with the typical nuclear morphology of apoptotic cells: nuclear condensation, budding of apoptotic bodies, and nuclear fragmentation (Fig. 4a). TNF alpha treated cells showed identical nuclear morphology to human sera treated porcine endothelial cells (Fig. 4b). Those porcine endothelial cells that did not have apoptotic nuclei exhibited apparently normal appearing nuclei. Greater than 90% of detached cells produced by treatment with human sera displayed apoptotic nuclei (Fig. 4c).

Propidium iodide counterstaining was used in conjunction with Ho258 to assess membrane integrity, since cells in the early stages of apoptosis have intact cell membranes. This is in contrast to porcine endothelial cells that have been killed by necrosis or porcine endothelial cells in the later stages of apoptosis which have undergone secondary cell lysis. Porcine endothelial cells containing apoptotic nuclei did not stain with PI (Fig. 4a-c). Necrotic cells killed by freeze/thawing or by heating at 43 °C showed nuclear staining with PI without accompanying nuclear condensation or formation of apoptotic bodies (Fig. 4d). The PI staining of necrotic cells obscured the weaker fluorescence of Ho258. Thus, the staining characteristics and morphology of porcine endothelial cells treated

with human sera were consistent with an apoptotic cell fate, and were highly similar to those observed from porcine endothelial cells exposed to TNF alpha.

For electron microscopy (EM), the porcine endothelial cells were fixed for 1 h in 2% gluteraldehyde in 0.1 M Sorensen's phosphate buffer. Following rinsing with 0.1 M Sorensen's phosphate buffer, the porcine endothelial cells were post fixed with 1% aqueous osmium tetraoxide for 1 h and then briefly rinsed with distilled water before dehydration through a series of graded acetones. Following several changes of 100% acetone, the porcine endothelial cells were infiltrated with a 1:1 (v:v) mixture of acetone:epon araldite followed by two changes of epon araldite. The cells were then embedded in fresh epon araldite and polymerized overnight at 60°C. One micron thick sections were cut on a Reichert Ultracut E ultramicrotome (Leica Canada Inc., Willowdale, ON), and stained with 1% Toluidine blue for examination under the light microscope. Ultrathin sections were then cut from a representative block using the Reichert Ultracut E ultramicrotome. The sections were collected on copper grids and counterstained with uranyl acetate and Sato's lead citrate. The porcine endothelial cells were examined at 60 KV on a Philips 400T transmission electron microscope (Eindhoven, The Netherlands) and representative photographs were taken. The porcine endothelial cells treated with human sera had ultrastructural features of apoptosis such as nuclear condensation, DNA clumping, margination of the clumped DNA to the nuclear membrane, dilation of endoplasmic reticulum, and nuclear clefting (Figs. 5a and b). In addition, there were apoptotic bodies formed by budding of the nuclear membrane (Fig. 5c). The cell membranes were usually intact, but in some cells there was blebbing of the cellular membrane. These findings on EM are pathognomonic of apoptosis (Sen S., Biological Reviews of the Cambridge Philosophical Society, 67(3): 287-319 (1992)). TNF alpha treated porcine endothelial cells also showed these features of apoptosis. Non-apoptotic porcine endothelial cells had normal cellular and nuclear morphology. There were also examples of apoptotic cells undergoing phagocytosis by adjacent cells (Fig. 5d), and examples of cells undergoing lysis. Since the nuclear morphology of the lysed cells have some features of apoptosis, these cells could represent

apoptotic cells undergoing secondary lysis.

Apoptotic porcine endothelial cells have decreased DNA fluorochrome stainability that appears as a sub-G1 population in DNA histograms (Darzynkiewicz Z., et al., Cytometry, 13(8): 795-808 (1992)). Although FACS analysis of nuclei to quantify apoptosis is commonly performed on lymphocytes, thymocytes, and on tumour cells, it has also been employed with endothelial cells (Polunovsky V.A., et al., Experimental Cell Research, 214(2): 584-94 (1994)). Prior to FACS analysis of nuclei, porcine endothelial cells were incubated with human sera or porcine sera, and sampled at the indicated times in Figure 6 and trypsinized (Gibco BRL). The porcine endothelial cells were washed once with PBS then fixed at -20°C with 70% ethanol overnight. The porcine endothelial cells were treated with solution A (3 mM trisodium citrate $2H_2O$, 0.1% NP-40, 1.5 mM spermine tetrahydrochloride, 0.5 mM Tris at pH 7.6) (Sigma) for 10 min, then with solution A and trypsin inhibitor and ribonuclease A for 10 min. Finally, PI and spermine tetrahydrochloride were first added to solution A and then to the porcine endothelial cells on ice for 1 h. FACS was performed on a single laser Coulter Profile I series flow cytometer (Coulter Electronics, Hialeah, FL) equipped with an argon ion laser (488 nm). Propidium iodide was detected after transmission through a 488 dichroic mirror, a 457-502 nm long pass blocking mirror, a 550 nm long pass dichroic mirror, a 600 nm short band dichroic mirror, and filtered with a 575 nm band pass filter (Vindelov L.L., et al., Cytometry, 3(5): 332-9 (1983)). The flow cytometry data was analyzed by Modfit II software (Verity Software House, Topsham, MA). Staining of treated cells with PI was carried out to confirm a hypodiploid population of cells indicative of apoptosis and to enumerate the apoptotic porcine endothelial cells. When porcine endothelial cells were cultured with porcine sera or with heat-inactivated human sera there was no increase in the number of hypodiploid cells. However, non-heat inactivated human sera induced the time-dependent emergence of a hypodiploid cell population (Fig. 6a) that progressively increased to 12% of the total cell population at 18 h, and then declined to 2% at 48 h. Cells treated with human sera showed an increase in proportion of cells in S phase from 7.4% at 18 h to 19.6% at 48 h, and this was

followed by an increase in the proportion of cells in the G2/M phase. Cells treated with TNF alpha also had hypodiploid cells (Fig. 6b). This pattern was not observed in those cells treated with porcine sera (Fig. 6c) or with heat-inactivated human sera which produced similar DNA histograms. In contrast, DNA histograms from necrotic cells induced by freeze/thawing showed a normal histogram without a hypodiploid population. Although there have been reports of DNA degradation during necrosis, this was not detected here, either because there was not enough time for DNA degradation to occur or because the necrotic cells were lost during processing for FACS.

To examine whether complement had an effect on porcine endothelial cell apoptosis, exogenous complement in the form of guinea pig sera was added to heat-inactivated human sera, and porcine endothelial cell membrane damage was determined by dual staining with two indicators of membrane integrity: CAM (retained by cells with intact membranes) and EHD (excluded by cells with intact membranes). At 1 h, heat inactivated human sera produced 3.7±1.0% of cells with membrane damage (Fig. 7a). Addition of guinea pig sera to heat-inactivated human sera increased the percentage of membrane damaged porcine endothelial cells to 22.1±2.7% (Fig. 7c). After 24 h of incubation, heat inactivated human sera produced 1.6±0.8% (Fig. 7b) of porcine endothelial cells with membrane damage, and after 24 h incubation of heat-inactivated human sera supplemented with guinea pig sera showed a decrease to 8.62±3.4% (Fig. 7d) of porcine endothelial cells with membrane damage.

Examination of these cells with Ho258 and PI demonstrated normal nuclear morphology in all groups at 1 h, regardless of whether PI was taken up. Heat-inactivated human sera supplemented with guinea pig sera did not produce an increased number of cells with apoptotic features even after 24 h of incubation (Figs. 7e and f), compared to 1 h of incubation. This indicates complement was an insufficient stimulus for the induction of endothelial cell apoptosis.

According to this example, porcine endothelial cells treated with human sera detached from the culture plates and exhibited morphological features of apoptotic cells such as shrinkage, membrane blebbing, nuclear condensation and formation of apoptotic bodies. These morphological findings were also apparent in the adherent porcine endothelial cells. Additionally, there was biochemical evidence of apoptosis in these cells such as DNA laddering on agarose gels and positive staining at the single cell level with the TUNEL method. There was also ultrastructural evidence of apoptosis, including nuclear condensation, DNA clumping, margination, nuclear clefting, and endoplasmic dilation in association with intact cellular membranes. Further, there were also isolated examples of cells undergoing phagocytosis by neighbouring cells, a phenomenon not associated with necrosis (Eastman A., Toxicology and Applied Pharmacology, 121(1): 160-4 (1993)). FACS analysis of cells treated with human serum also demonstrated a hypodiploid cell population indicative of apoptosis.

Supplementation of heat-inactivated human sera with exogenous complement was shown to produce cellular membrane damage of porcine endothelial cells. However, porcine endothelial cells treated with heat-inactivated human sera and exogenous complement at 24 h had decreased numbers of cells with membrane damage compared to those observed at 1 h. This is contrary to the results predicted by the current paradigm of xenotransplantation rejection in which the frequency of porcine endothelial cells with membrane damage would be expected to remain constant or increase with time. One possible explanation for this observation may be that necrotic cells were lost during the staining process, and were thus not detected at a later time. This is unlikely, however, since the density of cells remained the same after 24 h and the lysed cells were likely not replaced because DNA histograms and fluorescence microscopy show that the mitotic rate was insufficient to compensate for such a large number of damaged cells. In addition, there was no significant increase in the number of detached cells. This suggests that membrane damage produced by complement is reversible and is consistent with reported findings (Stahl G.L., et al., Circulation Research, 76(4): 575-83 (1995); Van den Berg C.W., et al., Journal of Immunology, 152(8): 4095-101 (1994); Benzaquen L.R., et al., Journal of

Experimental Medicine, 179(3): 985-92 (1994)). The implication of this finding is that strategies to defeat complement activation will not be sufficient, by themselves, to prevent xenograft rejection, since apoptosis will occur subsequently.

The peak rate of 12% endothelial cell apoptosis produced by human sera, as shown in this example, may have an important impact on graft survival in vivo, particularly if there was a continuing apoptotic stimulus coupled with the fact that apoptotic cell death may occur in as short a time as 2-3 h (Araki S., et al., [published erratum appears in Biochem Biophys Res Commun, Jun 29, 169(3): 1248 (1990)] Biochemical and Biophysical Research Communications, 168(3): 1194-299 (1990); Bursch W., et al., Biochemistry and Cell Biology, 68(9): 1071-4 (1990)). Endothelial cells apparently undergo a reversible antibody/complement injury that is conventionally thought of as endothelial cell lysis in the currently accepted model of xenotransplant rejection. It is unlikely that a significant number of cells were killed by antibody/complement for the reasons outlined above. Therefore, most of the cell death in this *in vitro* system can be attributed to apoptosis.

This example suggests that there are heat labile human factors that are not part of the described antibody/complement cascade which contribute to the manifestations of hyperacute rejection. It is not likely that the apogen is a Fas ligand, since endothelial cell Fas antigen ligation has been shown not to produce apoptosis in endothelial cells (Richardson B.C., et al., European Journal of Immunology, 24(11): 2640-5 (1994)), nor is TNF alpha a likely candidate because the kinetics of xenogenic apoptosis are different from those associated with TNF alpha treatment, and the concentration of TNF alpha in normal human sera is lower than that which is required to induce apoptosis.

Characterization of the apoptotic pathway occurring during xenograft rejection has important implications, since apoptosis is a regulated process and may be amenable to manipulation by molecular biology techniques. Delineation of a novel pathway of apoptosis in this context may provide further general understanding of apoptotic

mechanisms. Finally, identification of a human apogen will produce more specific therapy for treatment of discordant xenograft rejection.

Example 2: Induction Of Porcine Endothelial Cells Apoptosis By Human Serum Lectins

Porcine endothelial cells were prepared as described in Example 1.

Carbohydrate binding proteins were affinity isolated from human sera using several carbohydrate columns. DS-OSM was prepared as follows: frozen sheep submaxillary glands (Pet-Freez) were thawed and washed with cold water. The glands were pureed for 2 min with 0.01m Tris-HCl and stirred overnight at 4°C. After centrifuging (30 min at 5000 rpm with a JA-14 rotor, Beckman), the supernatant was filtered with cheese cloth and lyophilized. One gram of OSM was added to 50 ml of 0.1 N $H_2SO_4$ at 80°C for 1 h and then cooled, neutralized with 10 N NaOH to pH 7, and dialyzed against water (6-8,000 MW bag) overnight. The concentrations of GalNAc and other sugars were determined after acid hydrolysis of mucin by high pH anion exchange chromatography with pulsed amperometric detection.

The DS-OSM was coupled to cyanogen bromide activated sepharose fast flow (Pharmacia, Uppsula, Sweden) according to the manufacturer's instructions. Different batches of DS-OSM fast flow were prepared with ligand concentrations ranging from 2-25 mg/ml of gel. The presence of GalNAc on the fast flow resin was confirmed by high pH anion exchange chromatography with pulsed amperometric detection (Dionex) chromatography after hydrolysis of a test aliquot.

All reagents were from Sigma Chemical unless otherwise noted. Carbohydrate columns consisted of papGal, melibiose, GlcNAc, L-fucose, lactose, mannose, N-(e-aminocaproyl)-beta-D-galactopyranosylamine, N,N'-diacetylchitobiose, GalNAc coupled individually to 4% beaded agarose matrix and the DS-OSM column. Human

sera was applied to the columns. The columns were washed extensively with 100 mM Tris-HCL buffer, pH 7.4, to remove any unbound protein. Samples of washed buffer were collected and checked for unbound protein using the Bio-Rad protein assay (Bio-Rad Laboratories). Elution buffer for the papGal, melibiose and GlcNAc columns consisted of 100 mM Tris-HCl, pH 7.5 and 1 M D(+) galactose. L-fucose, melibiose, GalNAc, lactose, mannose, N,N'-diacetylchitobiose, and N-(e-aminocaproyl)-beta-D-galactopyranosylamine columns were eluted with 1 M of their respective carbohydrates in Tris buffer, pH 7.5. A Sepharose 4B-CL column (Pharmacia, City, Sweden) (the agarose matrix without carbohydrate) served as a negative control. The Sepharose 4B-CL column was eluted with glycine buffer to determine whether human serum proteins non-specifically bound to the resin without carbohydrate ligands. The DS-OSM column was eluted sequentially with 7.5 mM EDTA, 1 M galactose and 125 mM GalNAc in Tris buffer. Elution was carried out until fractions did not contain protein as determined by the Bio-Rad protein assay. The fractions which contained protein were pooled and concentrated using Centricon 3 concentrators (Amicon, Missisagua, ON). A greater amount of serum protein was bound to papGal, melibiose, DS-OSM, and GlcNAc columns than to L-fucose, lactose, and mannose columns. However, no protein was bound to N-(e-aminocaproyl)-beta-D-galactopyranosylamine, N,N'-diacetylchitobiose, GalNAc columns or the resin beads (Sepharose).

The isolated proteins were subjected to SDS-PAGE. There were multiple bands in the proteins eluted from the papGal, melibiose and DS-OSM columns including bands with the same molecular weight as antibodies such as IgM and IgG. Proteins isolated from the papGal column were subjected to DEAE chromatography.

Ion exchange chromatography was used for further purification because antibodies have a more basic isoelectric point than the majority of other serum proteins, and can also bind carbohydrates. A 2 ml column of DEAE-Sepharose was used. The column was washed and equilibrated with 5 mM phosphate buffer, pH 6.5. Five milliliters of concentrated papGal proteins were applied to the column. The column was washed

with 10 bed volumes of 5 mM phosphate buffer, pH 6.5. The column was then eluted with increasing NaCl concentrations by step buffers (i.e. three column volumes of 50 mM NaCl in 5 mM phosphate buffer, followed by three column volumes of 100 mM NaCl in 5 mM phosphate buffer, sequentially up to 1M NaCl concentration). Fractions were monitored by measuring their optical density at 280/260 and using the Bio-Rad protein assay. The fractions were then analyzed using reducing and non-reducing SDS-PAGE and Coomassie Blue staining with Novex See-Blue markers (Novex, San Diego, CA) and IgG and IgM standards.

DEAE chromatography produced four fractions, one of which was a single band on SDS-PAGE (Fig. 8). The molecular weights of the proteins purified by DEAE chromatography were 73 kD, 60 kD, 32 kD and 33 kD. The 73 kD protein eluted using 200 mM NaCl was a single band on reducing SDS-PAGE.

The detachment apoptosis assay was performed as follows: porcine endothelial cells were cultured to confluency in 96 well plates (Nunc). The cells were then incubated for 18 h at 37°C with papGal binding serum proteins, the 73 kD papGal isolate, TNF alpha (40 ng/ml), SBA, WGA, BSI4, MPA, TPP, PNA (EY Laboratories, San Mateo, CA), and human IgM diluted in medium. Human IgM was purified from human serum using an IgM purification kit according to manufacturer's instructions (Pierce, Rockford, IL). The cells were washed a final time with PBS and then 100 µl of 5 mM CAM was added to the cells for 30 min. The CAM solution was poured off and the plate was scanned with a Millipore Cytofluor 2300 fluorescence plate scanner (Millipore, Bedford, MA) (excitation 485 nm, emission 530 nm). Detachment rate was calculated as a percentage of CAM fluorescence of cells treated with medium alone.

Porcine endothelial cells incubated for 4 h with human carbohydrate-binding proteins isolated from the papGal column underwent apoptosis. The porcine endothelial cells exhibited blebbing, shrinking, and detachment from the culture plate. Fluorescence microscopy (as described in Example 1) revealed typical features of apoptosis, both

in cells remaining adherent to the culture dish and in those cells which detached from the culture dish, including nuclear condensation, formation of apoptotic bodies, and nuclear fragmentation. Greater than 90% of detached cells were apoptotic compared to 4.7±1.2% of adherent cells when examined with the TUNEL assay or 3.4±1.1% of cells when examined with Ho258 staining. Neither detached nor adherent cells exhibited increased PI uptake (Fig. 9). Likewise, proteins isolated using the fucose, mannose, lactose, melibiose, DS-OSM and GlcNAc columns also produced porcine endothelial cells apoptosis.

The number of detached cells was used as an indicator of apoptosis in a fluorescence plate reader assay based on the finding that almost all detached cells were apoptotic and were not necrotic. The papGal proteins isolated from human sera caused up to 90% porcine endothelial cell detachment, as did the 73 kD papGal isolate (Fig. 10). Likewise, the isolates from the melibiose column caused approximately the same amount of detachment. This concentration of the melibiose lectins and the 73 kD papGal isolate were below the limits of detection of the Bio-Rad protein assay. In comparison, human IgM produced only 30% detachment. TNF alpha produced 30% detachment, which is consistent with other studies which show a similar rate of apoptosis (Robaye B., et al., American Journal of Pathology, 138(2): 447 (1991)).

To further define the targets of human carbohydrate binding proteins, plant lectins with known specificities, shown in Table 2, were used in the cell detachment assay to determine other potential xenogenic epitopes. The lectin BSI4 produced a dose-dependent cellular detachment rate of 70% (Fig. 11), which was inhibited by galactose. Wheat germ agglutinin and SBA were also effective in producing cell detachment (Fig. 11). Several lectins did not produce porcine endothelial cell death: MPA, TPP, and PNA, even when 1 mg/ml of the lectin was used. Plant lectin-induced apoptosis was confirmed by Ho258 staining of detached cells.

**TABLE 2**
**Plant lectin specificities**

| | | |
|---|---|---|
| BSI4 | Bandeiraea simplicifolia | alpha D-Gal |
| CSA | Cytisus scoparius | alpha /beta Gal,GalNAc |
| UEA-I | Ulex europaeus | alpha L-Fucose |
| RCA-I | Ricinus Communis | beta Gal> alpha Gal>GalNAc |
| WGA | Triticum Vulgare | (beta (1,4) GlcNAc)2> beta GlcNAc>NeuAc oligosacharides |
| PHA | Phaseeolus vulgaris | alpha-Man> beta Glc |
| ConA | Concanavalia ensiformis | GalNAc alpha (1,3) D- GalNAc> alpha GalNAc |
| DBA | Dolichos biflorus | alpha D-Glc, alpha Man |
| LCA | Lens culinaris agglutinin | Sialic acid alpha (2,3) galactose |
| MAA | Maackia amurensis | alpha /beta GalNAc |
| SBA | Glycine max | alpha Man |
| PSA | Pisum sativum | Gal or GalNAc |
| MPA | Maciura pomifera agglutinin | Gal beta (1,3) GalNAc |
| PNA | Peanut agglutin | |

The significance of the Gal alpha epitope in PAEC apoptosis can be investigated by enzymatically removing alpha linked Gal residues and measuring the rate of apoptosis induced by human serum. PAEC, grown in a microtitre as described in Example 1, were treated with alpha-galactosidase (which is specific for both alpha 3 and alpha 6 linked Galactose residues, Boehringer Manneheim). The stock solution of alpha galactosidase was centrifuged at 10,000 xg for 30 min at 4°C, the supernatant was removed and then the enzyme was reconstituted with 4% BSA-Earls Balanced Salt Solution (EBSS) at pH 7.2. The cells were treated with the enzyme for 3 h at 25°C. The wells were emptied and then 30% human AB serum was added for 20 h. The detachment apoptosis assay was performed as described above. The PAEC treated with 0.25mg/mL of alpha galactosidase and then human sera had a detachment rate of 12.6% compared to a detachment rate of 32.1% for cells that were not treated by the enzyme. This represents a 61% reduction in the apoptosis rate as detected by the apoptosis detachment assay and indicates that the Gal alpha epitope is involved in human serum lectin mediated apoptosis of PAEC.

This example demonstrates that carbohydrate ligation by endogenous human lectins can mediate apoptosis in untransformed cells. The lectins did not disrupt porcine endothelial cell membranes as demonstrated by exclusion of PI, but rather, produced morphological and biochemical signs of apoptosis (Sen S., Biological Reviews of the Cambridge Philosophical Society, 67(3): 287 (1992); Ueda N., et al., Journal of Laboratory and Clinical Medicine, 124(2): 169 (1994)). These include nuclear changes such as nuclear condensation, apoptotic body formation, and nuclear destruction, and cellular changes such as blebbing, cell body shrinkage, and cell detachment from the extracellular matrix. *In situ* labelling by TUNEL demonstrated endonuclease activity, which is characteristic of most apoptotic processes. One of the consequences of apoptosis is phagocytosis, either by professional phagocytic cells such as neutrophils and macrophages, or by adjacent endothelial cells (Fadok V.A., et al., Journal of Immunology, 149(12): 4029 (1992); Haslett C., Clinical Science, 83(6): 639 (1992); Hall S.E., et al., Journal of Immunology, 153(7): 3218 (1994)). The majority of apoptotic cells do not undergo phagocytosis but subsequently detach into the surrounding extracelluar space. The number of detached cells may be estimated by determining the number of attached cells relative to control samples as indicated by metabolic live dye uptake.

Since it has been hypothesized that the anti-Gal-alpha-3Gal antibody is the predominant xenogenic antibody, we examined whether there might be a corresponding xenogenic lectin which might recognize Gal alpha epitopes. This hypothesis was verified by the demonstration that papGal binding proteins derived from DEAE chromatography, which were not antibodies, produced porcine endothelial cell detachment indicative of apoptosis. N-acetyl glucosamine binding proteins also produced apoptosis as well as proteins binding to fucose, melibiose, mannose, and lactose. No proteins bound to the GalNAc columns, but there were proteins purified on the DS-OSM column (a mucin rich in GalNAc). The likely explanation is that GalNAc by itself is not recognized by lectins or antibodies, but requires conjugation to a peptide for binding, as demonstrated by protein binding observed with the the DS-OSM column.

In summary, this embodiment of the present invention demonstrates the presence of xenogenic lectins in human sera directed against porcine endothelial cells which produce endothelial cell apoptosis. The lectins were isolated using affinity and ion exchange chromatography and exhibited the same specificity as plant lectins directed toward the same epitope. The apoptosis assay of the present invention was used to systematically disclose the presence of specific, functionally significant xenogenic epitopes, including Gal alpha, GlcNAc, and GalNAc.

Example 3: A Method for Detecting Human Serum Induced Porcine Endothelial Cell Apoptosis

PAEC were grown in microtitre plates as described in Example 1. Human AB sera (HS) (from the hospital blood bank) was diluted to 10, 20, 30, 40, and 50% solutions with M199. TNF alpha (Calbiochem, La Jolla, CA) was diluted to 8, 16, 24, 32, and 40 ng/ml with M199. The PAEC cultures were incubated for 18 h at 37°C with either HS or TNF alpha.

After 18 h of incubation, detached cells (floating cells and cells dislodged by additional washing) and trypsinized adherent cells were collected separately and fixed immediately at -20°C with ethanol for 10 min. To assess nuclear morphology, the fixed cells were washed with PBS, allowed to stick to glass slides and air dried. The cells were then washed with PBS and stained with 100 µg/mL Ho258 for 30 min at room temperature.

For the detection of cleaved DNA *in situ*, the cells were fixed in 4% neutral buffered formalin for 10 min, allowed to air dry on glass slide and post-fixed with an ethanol:acetic acid 2:1 (v:v) solution at -20°C for 5 min. The ApopTag Kit (Oncor, Gaithersburg, MD) was used to terminal deoxynucleotidyl transferase label the 3'-OH ends of cleaved DNA with digoxigenin-dUTP and the digoxigenin was detected by anti-digoxigenin-fluorescein. The stained cells were then examined with

a fluorescence microscope (Olympus, AH3) equipped with mercury lamps epi-illumination and a 440 - 460 nm excitation filter (Olympus, Carsen Medical, Markham, ON). Two different filters were used: 420 nm (for the TUNEL assay) and 570 nm (for the Hoechst 33258). Photographs were taken and all slides were digitized.

The quantitation of apoptosis was performed by enumeration of either the cells showing apoptotic nuclear features after staining with Hoechst 33258 or the cells positively labelled by TUNEL assay. Greater than 500 cells were counted for each of the triplicate experiments.

PAEC were cultured to confluency on 96 well plates (Linbro, McLean, VA). The cells were then incubated for 18 h at 37°C with either HS, TNF alpha, or M199. The cells were washed twice with PBS and then 100 µl of 2.5 mM Calcein AM (Molecular Probes, Eugene, OR ) was added to the cells for 30 min at 37°C. The Calcein AM solution was poured off and the plate was scanned with a fluorescence plate reader (485 nm excitation, 530 nm emission; Cytofluor 2300 Millipore, Bedford, MA). Detachment rate for the sample of interest was calculated as a percentage based on Calcein AM fluorescence of cells treated with media only.

TUNEL assay was performed on PAEC and analyzed by flow cytometry. PAEC were incubated with various concentration of HS or TNF alpha for 18 h, and all the cells were collected. The cells were pre-fixed with 2% paraformaldehyde for 15 min on ice, washed and fixed at -20°C with 70% ethanol overnight. Fixed cells were permeabilized with 0.1% Triton X-100 for 5 min, and then labelled by TUNEL following the manufacturer's instruction, but without counter staining with propidium iodide. Flow cytometry was performed on a single laser Coulter Profile I series flow cytometer (Coulter Electronics, Hialeah, FL) equipped with an argon ion laser (488 nm). Green fluorescence of anti-digoxigenin-fluorescein was detected after transmission through a 488 nm dichroic mirror and passage through a 457-502 nm long pass blocking mirror, a 550 nm long pass dichroic mirror, and then a 525 nm

band pass filter. For all FACS analysis, over 10,000 cells were counted.

PAEC were incubated with various concentrations of HS or TNF alpha for 18 h, and all the cells were collected. The cells were washed once with PBS then fixed at -20°C with 70% ethanol overnight. The cells were treated with solution A (3 mM trisodium citrate $2H_2O$, 0.1% NP40, 1.5 mM spermine tetrahydrochloride, 0.5 mM Tris at pH 7.6) (Sigma, St. Louis, MO) for 10 min, then with solution B (trypsin inhibitor and ribonuclease A) for 10 min. Propidium iodide and spermine tetrachloride were added to solution A and then added to the cells on ice for 1 h. FACS was performed as described above and propidium iodide fluorescence was detected after transmission through a 488 nm dichroic mirror and passage through a 457-502 nm long pass blocking mirror, a 550 nm long pass dichroic mirror, a 600 nm short band dichroic mirror, and a 575 nm band pass filter.

The linearity of all data sets was determined by regression analysis, and ANOVA was performed to determine correlation and significance. The slopes and intercepts of different curves were compared with ANOVA. The P values are reported and $p<0.05$ was considered significant.

When PAEC were treated with 25% HS for 18 h, some cells underwent detachment from their underlying extracelluar matrix. The detached cells exhibited nuclear morphology characteristic of cells undergoing apoptosis. The detached cells were smaller than the attached cells and contained bright granule-like nuclei and apoptotic bodies when observed by inverted phase contrast microscopy. Cells which were loosely attached were easily dislodged by washing. In contrast, adherent cells typically displayed normal nuclear morphology except those that had features of cells in the early stages of apoptosis such as nuclear condensation. There were no necrotic cells observed.

The adherent and detached cells were analyzed by fluorescence microscopy after staining with chromatin dye Ho258 to determine nuclear morphology (Figure 12a and

12b). Detached cells were apoptotic at a rate of 92.8±2.0% based on either chromatin condensation or nuclear fragmentation. In contrast, only 10.5±2.2% of adherent cells were apoptotic. The TUNEL assay was performed to confirm the results of the morphological assay (Figure 12c, and 12d). Detached cells were apoptotic at a rate of 98.2±0.7% and 5.7±0.5% of adherent cells were TUNEL positive.

The detachment assay was compared to two established apoptosis assays: the FACS-TUNEL assay (Gorczyca W., et al., Cancer Research, 53:1945-51 (1993); Li X., et al., Leukemia & Lymphoma, 1:65-70 (1994); Gold R., et al., Laboratory Investigation, 71:219-25 (1994)) and the FACS-DNA assay (Darzynkiewicz Z., et al., Cytometry, 13:795-808 (1992)). The apoptosis detachment assay of the present invention, FACS-DNA, and the FACS-TUNEL assay demonstrated a linear dose-dependent measurement of PAEC apoptosis during 18 h of exposure to the apogen, TNF alpha (Fig. 13a). A uniformly higher percentage of apoptosis was detected in the TUNEL and detachment assays as compared to FACS-DNA. There was significant correlation ($p<0.05$) between the TNF alpha concentration and percentage apoptosis or percentage detachment as determined by ANOVA for the TUNEL ($r^2=0.925$) and detachment assay ($r^2=0.987$), respectively. This did not reach significance in the case of FACS-DNA ($P=0.09$, $r^2=0.649$). Exposure of PAEC to HS demonstrated results similar to that of TNF alpha (Fig. 13b), although in this case, all three assays showed linear dose-dependent relationships as well as significant correlation ($p<0.05$) ($r^2=0.929$, $r^2=0.864$, and $r^2=0.986$ for the TUNEL, FACS-DNA, and detachment assays respectively) between HS concentration and percentage apoptosis or percentage detachment.

To determine whether endothelial cell detachment could be correlated with apoptosis as determined by the TUNEL assay, the data for Figures 13a and 13b were analyzed by graphing PAEC detachment against FACS-TUNEL utilizing TNF alpha or HS as the apogen (Fig. 14). There was a significant positive correlation between apoptosis as estimated by TUNEL and by detachment for both TNF alpha treatment ($P = 0.006$,

$r^2$=0.938) and HS treatment (P = 0.04, $r^2$=0.796). Similar results were found based on the comparison of the detachment assay with FACS-DNA using TNF alpha (P=0.02, $r^2$=0.887) and HS (P=0.10, $r^2$=0.804).

Endothelial cells undergo a number of morphological and biochemical changes during apoptosis. Porcine endothelial cells cultured with TNF alpha, a known apogen, or with HS acquire a spherical shape, in comparison to their normal flat cobblestone shape, and show surface blebbing when observed by inverted phase contrast microscopy. Electron microscopy typically shows condensed DNA associated with margination along the nuclear membrane, nuclear clefting, cellular blebbing, and formation of apoptotic bodies. Nuclear staining with DNA fluorochromes also reveals some of these features such as nuclear condensation, blebbing and apoptotic body formation. As cells undergo apoptosis, they exhibit decreased propidium iodide DNA stainability attributable to a loss of DNA and detectable by FACS analysis (Fraker P. J., et al., Methods in Cell Biology, 46:57-76 (1995); Polunovsky V.A., et al., Experimental Cell Research, 214:584-94 (1994)). The activation of an endonuclease which cleaves chromatin in 120-200 bp repeats produces a biochemical change frequently associated with apoptosis and is revealed by DNA laddering on an agarose gel, or positive staining by the TUNEL assay (Gavrieli V., et al., Journal of Cell Biology, 119: 493-501(1992)). Furthermore, endothelial cells also detach from their underlying extracellular matrix during apoptosis.

Human sera exhibit considerable variability in their ability to kill pig (PK-15) cells as assessed by various cytotoxic assays (Kujundzic M., et al., Xenotransplantation, 1:58-65 (1994)). Despite this variability in vitro, untreated xenograft rejection in this discordant model occurs invariably within 90 minutes of reperfusion. One possible explanation for this discrepancy suggested by the experiments discussed herein is that cytotoxicity assays do not account for apoptotic cell death, and thus underestimate the extent of xenogenic-mediated cell loss.

Fluorescence microscopy of PAEC treated with HS demonstrates that most of the detached PAEC exhibited apoptotic morphology such as condensed chromatin,

apoptotic bodies, or nuclear blebbing (Figs. 12a and 12b). In addition, detached cells stained positively for apoptosis using the TUNEL method (Figs. 12c and 12d).

Porcine endothelial cells undergo apoptotic cell death upon exposure to human sera based on several different morphological and biochemical criteria. In the previous examples, it was shown that carbohydrate-binding proteins isolated from human sera that are distinct from classical antibodies, i.e., lectins, produce apoptosis of PAEC accompanied by cell detachment from the extracelluar matrix. Endothelial cells, as well as other adherent cell types, have been shown to undergo apoptosis when cell adhesion is disrupted, either by mechanical means (Malorni W., et al., Biochemical & Biophysical Research Communications, 207: 715-24 (1995); Re F., et al., Journal of Cell Biology, 127:537-46 (1994), or by anti-adhesion protein antibodies (Meredith et al., Molecular Biology of the Cell 4: 953-61 (1993)). It is not clear whether human lectins and antibodies produce apoptosis directly, or promote detachment and then apoptosis. Nevertheless, detachment is intimately associated with endothelial cell apoptosis and can be used as a physiologic endpoint in an assay to detect apoptosis.

The number of detached cells may be estimated by determining the number of remaining attached cells by measurement of metabolic live dye uptake compared to a control. Exposing PAEC to increasing concentrations of TNF alpha and HS demonstrated a positive linear dose-dependent relationship between the concentration of apogen and percentage apoptosis as measured by FACS-TUNEL or FACS-DNA and that measured by the detachment assay (Figs. 13a and 13b).

In estimating the rate of apoptosis based on cell detachment, there exists the possibility that the detached cells may also include cells which die by necrosis or are yet still viable. Further, the washing process required in this assay may dislodge viable cells and likewise contribute to an overestimation of the rate of apoptotic cellular detachment. However, our results demonstrate that greater than 90% of detached cells were apoptotic. Dual staining of detached cells with Ho258 and

among the adherent cells at 18 h. These adherent apoptotic cells may be in early stages of apoptosis, or they may represent apoptotic cells undergoing phagocytosis. Adherent apoptotic cells contribute to underestimation of the apoptotic rate, since they are notenumerated by the detachment assay. These events are infrequent, however, and on balance do not appreciably detract from the quantitative validity of the detachment assay.

Other cell types such as cancer cell lines (e.g. T47D and Calu-3) and normal human cells (primary cultured preadipocytes, human pulmonary arterial endothelial cells, human aortic endothelial cells) also undergo detachment upon exposure to apogens. The detached cells were apoptotic as shown by TUNEL staining and had apoptotic nuclear morphology as shown by Ho258 staining.

The detachment assay represents a powerful screening assay for anti-apoptotic compounds for test systems in which the apogen has been validated previously. This assay provides a rapid, accurate and quantitative assessment of apoptosis as determined in relation to an established quantitative assay of apoptosis (FACS-TUNEL or FACS-DNA). Thus, the detachment assay provides a simple, rapid, and inexpensive alternative to the traditional quantitative methods of analysis of apoptosis. The utility of the apoptosis detachment assay for screening combinatorial libraries is further shown in the Examples 7 and 8.

Example 4: DS-OSM-Binding Proteins and Apoptosis of Cancer Cells

T47D cells (human breast ductal carcinoma cells. ATCC, Rockville, MD) were cultured in plastic T-flasks (85cm2, Nunc, Naperville, IL) in 640 Medium (Life Technologies, Grand Island, NY) with 10% fetal bovine serum (Immunocorp, Montreal, QE), 0.2 IU/ml bovine insulin (Sigma, St. Louis, MO.), and 1% antibiotic-antimycotic (penicillin G, streptomycin and amphotericin B, Life Technologies) in 5% $CO_2$ at 37°C. Calu-3 cells (human lung adenocarcinoma cells. ATCC) were cultured in T-flasks in Eagle Minimun Essential Medium with 1mM

propidium iodide did not reveal any cells which were either membrane damaged, i.e., lysed, or did not exhibit apoptotic nuclear morphology upon exposure to human serum apogens. Lysed cells may not be detected by the staining of detached cells in the event the cells fragment to such a degree that the remnants are not stainable. That this is not the case is indicated by the observation that both freeze/thawed and detergent treated cells are stained by propidium iodide. The net overestimation of the apoptotic rate measured by the detachment assay compared to that determined by FACS-TUNEL and FACS-DNA is reflected in the slopes of regression lines which exceeded 1.0 (see Fig. 14 - range: 1.3-1.6) for both TNF alpha- and HS-mediated apoptosis. The correlation between detachment and percentage apoptosis as measured by TUNEL is highly significant for both TNF alpha and HS dose-response curves.

Many cytotoxicity assays depend on an assessment of cell membrane integrity to enumerate live versus dead cells (Charreau B., et al., Transplantation, 58:1222-9 (1994); Kennedy S. P., et al., Transplantation, 57:1494-501 (1994); Neethling F. A., et al., Transplantation, 57:959-63 (1994); Zhao Z., et al., Transplantation, 57:245-9 (1994).). These assays may not be appropriate for quantifying apoptosis since apoptotic cells maintain their membrane integrity until the late stages of apoptosis. Furthermore, because apoptotic cells lose their surface attachment, most of the cells are lost through the washing process required in most viability/cytotoxicity assays. This contributes to the underestimation of apoptotic rates by cytotoxicity assays. Other apoptotic assays, such as the TUNEL assay, and DNA content analysis, also tend to underestimate the true rate of apoptosis because of cell loss through processing. However, since cell detachment was used to estimate apoptosis in this study, cell loss through processing would tend to limit the degree of underestimation of the rate of apoptosis inherent in these other assays.

The possibility that not all apoptotic cells become detached would contribute to an underestimation of apoptosis as measured by detachment. Adherent cells may represent live cells, necrotic cells or apoptotic cells. The percentage of adherent cells that was apoptotic was 5.7-10.5%, whereas there were no necrotic cells detected

sodium pyruvate (Sigma) and 10% fetal bovine serum (Immunocorp). Cells were passaged every 4 days with 0.25% Trypsin-EDTA (Sigma). HAEC 2241-1 (human aortic endothelial cells) and HPAEC 2264 (human pulmonary arterial endothelial cells, Clonetics, San Diego, CA) were grown in EGM (modified MCDB131 media with 2% FBS, 12 µg/ml bovine brain extract, 1 µg/ml hydrocortisone, 0.01 ng/ml human epidermal growth factor, 0.05 mg/ml Gentamicin and 0.05 µg/ml Amphotericin B, Clonetics) in 5% $CO_2$ at 37°C. Both endothelial cell lines were passaged once every 10-14 days by using 0.025% Trypsin-EDTA (Clonetics). For microtire plate assays, cells were plated at a density of $7.5x10^3$ per 100 ul for T47D breast cancer cells and Calu-3 lung cancer cells and $4x10^3$ per 100 µl for endothelial cells for each well of a 96-well plate (Linbro, Aurora, ON).

Liposuction tissue, treated with hyaluronidase at the time of surgery, was transferred into Krebs/Ringer/Hepes (KRH) buffer (25ml of tissue/20ml KRH), and centrifuged at 500 xg for 5 min. The floating layers were removed and the process was then repeated twice. The remaining cells were treated with 10 ml KRH containing 4.4 mg/ml collagenase, 40 mg/ml bovine serum albumin (BSA) fraction V, 40 U/ml penicillin, and 50 mg/ml streptomycin for 1 h at 37°C with constant agitation. The cells were rinsed with Ham's/DMEM with 10% FBS (media containing penicillin and strepomycin) and centrifuged at 500 xg for 5 min. The free fat was removed and after further centrifugation, the floating adipocyte cells were transferred to 50 ml tubes containing 25 ml media and strained through mesh. The cells were either plated into 35 mm dishes or into 24 well dishes. Thirteen-mm Thermanox coverslips were placed over the adipocytes. The coverslips were inverted after the cells were attached and left in the incubator and washed with Ham's/DMEM with 10% FBS (media containing penicillin and strepamycin). The media was changed every other day. (Funatsumaru S., Cell Structure And Function, 20:23-32 (1992), Prins J.B., et al., Biochem Biophys Res Commun, June 15, 201(2): 500-505 (1994), Hazen S.A., et al., Journal Of Lipid Research, 36:868-875 (1995), Bjorntorp P., et al., Journal Of Lipid Research, 19:316-324 (1978)).

Proteins that bound to DS-OSM were isolated from human serum and eluted with GalNAc and concentrated (see Example 2). The proteins underwent SDS-PAGE with 8-16% gradient gel under reducing conditions for 30 min at 75 V and then 1.6 h at 12 V using 25 mM Tris, 192 mM glycine, and 0.1% SDS, pH 8 buffers (Laemmli U. K., Nature, 227: 680-5 (1970)). The transfer to PVDF membranes was carried out with 10mM CAPS buffer, pH 11.0 with 10% methanol for 30 min at 100 V constant voltage (Matsudaira P., Journal of Biological Chemistry, 262: 10035-8(1987)). The membranes were rinsed with distilled water and 20% methanol, and then with 100% methanol and stained with 0.1% Coomassie Blue R250 in 1% acetic acid/40% methanol for 30 s and then destained with 50% methanol. The stained protein bands were excised and transferred to an Applied Biosystems Blott Cartridge (P/N 401096) for automatic sequencing by Edman degradation chemistry (Brauer A. W., et al., Biochemistry, 14: 3029-35 (1975)) Seven major bands were sequenced (45kD-alpha 1 antitrypsin HQDHPTFDKITPD,DDQHPTFDKITP; 70kD-albumin NAHKSQVAHRFKBLGQQNF; 100kD alpha macroglobulin ...QYMVLV...L; 80kD IgG heavy chain EVQLVESGGGLVQPG and transferrrin VPD/SKTVRYM/SA/VVSSSE; 30kD IgG light chain NVLPPSPAVELLGGV and an unknown peptide I/LKSDALKSVSQGP; 60kD IgG heavy chain EVQLLVESGGGLVQPG and 40kD haptoglobulin ILGGHLDAKGSFPWQ).

cDNA cloning of human serum lectins could be carried out by the use of degenerate primers based on amino acid sequences of purified lectin, homology screening using conserved regions of known lectins, or expression cloning using labelled DS-OSM to detect clones expressing the lectin (Sambrook J., et al., Molecular Cloning: A laboratory Manual, 2nd ed. 13.3-13.53 Cold spring Harbor Lab Press. Cold Spring Harbor, NY (1989); Stuart A., et al., Methods in Neuroscience: Receptor Molecular Biology 5: 90-102 (1995)).

For cDNA cloning, a cDNA library from human liver (a likely site of lectin synthesis) can be obtained from Clontech (Palo Alto, CA). Based on the amino acid sequences of the lectin, two pairs of degenerate oligonucleotide primers (outer and nested pairs)

can be synthesized for use in PCR using the cDNA library as a template. The PCR product purified by preparative agarose gel electrophoresis can be subcloned and the sequence of selected inserts of clones can be determined by the dideoxynucleotide termination method. The generated nucleotide sequences can then be utilized to synthesize new primers to extend the sequence information. The full-length lectin cDNA could be isolated by the use of a large PCR probe. The expression of the lectin *in vitro*, in insect cells and in mammalian cells can be assayed by the use of labelled anti-lectin antibodies and by the use of labelled glycoprotein ligand. Once the full length cDNA is obtained, the gene encoding the lectin can be cloned, using a human genomic library. To express large amounts of recombinant protein, the cDNA encoding the lectin can be intitially expressed in a baculovirus/Sf9 insect cell system. (Toki D., et al., Biochemical J. in press, (1997)). Biological assays for recombinant lectin activity produced in insect cells (e.g. detachment apoptosis assay using T47D cells) can be carried out using the insect cells medium and the Sf9 cell pellet. Recombinant lectin can be purified as described above in Example 2 for comparison to the lectin isolated from human serum.

Human breast cancer T47D cells were incubated with the DS-OSM-binding protein fraction for 24 h. DS-OSM binding lectins produced 76% killing of T47D cells at 75 µg total protein/ml compared to cyclophosphamide (CPA) as a positive control, which produced the same amount of killing at 10 mg/ml (Fig. 15). PapGal, melibiose-binding human serum proteins and blank elutions produced less cytoxicity than human serum. At a concentration of less than 10 µg protein/mL, DS-OSM binding lectins produced 24% killing of T47D cells.

To examine whether plant lectins that also recognize GalNAc can mediate killing of T47D cells, Dolichos biflorus, lima bean agglutinin, Helix pomatia, Griffonia simplicifolia-IA4, soybean agglutinin (all recognizing GalNAc-), and Jacalin lectin (recognizing Gal beta (1,3) GalNAc) were incubated with T47D cells. Only soybean agglutinin showed T47D killing of 12% with a concentration of 300 µg/ml while the other lectins did not exhibit cell killing activity. The plant lectins, however,

inhibited proliferation of T47D cells, with SBA showing the most effect. To determine whether this was due to differential binding of these plant lectins, FITC-labelled lectins were used to stain T47D cells and assessed with a fluorescent microtitre plate reader. Interestingly, soybean agglutinin bound the least to the T47D cells compared to the other plant lectins. This implies that lectin binding to GalNAc is insufficient to produce cell killing, and suggests that the interaction between DS-OSM-binding proteins and T47D cells is more complex than simple lectin-carbohydrate binding.

An anti-sialyl-Tn antibody did not produce detachment of T47D cells and did not bind to T47D cells. The T47D cells were blood typed using both murine monoclonal antibodies and by a panel of plant lectins and shown to be blood type O and therefore not expressing a blood group related GalNAc. The cells which detached following exposure to DS-OSM binding lectins were positively stained by TUNEL (Oncor). This suggests that the epitopes recognized by DS-OSM binding lectins are non-sialylated, non-blood group GalNAc epitopes and have the capacity to trigger apoptosis.

To examine the binding specificity of DS-OSM binding lectins, galactosamine, mannose, and GalNAc were preincubated with DS-OSM binding lectins. GalNAc at a concentration of 5 mM, but not mannose or galactosamine, decreased DS-OSM-binding protein-mediated killing of T47D cells to 3%, representing an 88% reduction in killing capacity. This indicates that the isolated lectin indeed binds to GalNAc on T47D cells. Since a major class of serum lectins is represented by collectins, which are specific for mannose structures, the inability of mannose to inhibit T47D killing by DS-OSM-binding proteins implies the absence of collectin-mediated killing. The isolated DS-OSM binding lectins contained a mixture of proteins. Bands which corresponded to albumin, haptoglobin, alpha 1 antitrypsin, transferrin, IgG, and IgM were represented prominently. Purified fractions of these proteins did not produce cell detachment.

The specificity of DS-OSM binding lectins for cell type was also examined. DS-OSM binding lectins were tested against normal human cells. There was minimal toxicity against human adipocytes derived from primary tissue, human pulmonary arterial endothelial cells or human aortic endothelial cells obtained from commercial sources (Clonatech, CA). Calu-3, a blood group A-expressing lung adenocarcinoma cell line (typed with murine monoclonal antibodies), was also resistant to DS-OSM binding lectin-mediated apoptosis in cells to which the plant lectin LBA (specific for GalNAc) did not bind, but were susceptible to DS-OSM binding lectins in cells to which LBA did bind. Calu-3 cells were incubated with DS-OSM binding lectins at various concentrations for 24 h and cytotoxic activity was quantified using the detachment apoptosis assay. Thirty µg/ml of DS-OSM binding lectins produced 98% cells detachment of Calu-3 cells (Fig. 16). LBA and DBA (300 µg/ml), pap-Gal lectin (200 µg/ml, not shown) and control buffer from the column had very low cytotoxic effects on Calu-3 cells in this assay. For histochemical analysis, in-situ nick end labeling assay was performed on Calu-3 cells treated with 7 µg/ml of DS-OSM binding lectin. Greater than 95% of cells that were detached from the culture dish showed the presence of fragmented DNA, indicating that these cells underwent an apoptotic death. Cyclophosphamide(CPA) produced non-specific detachment of Calu-3 and T47D cells. *In vivo*, CPA is metabolized into an active compound (4-hydroxycylophosphamide) that has cancericidal properties. These experiments indicate that DS-OSM binding lectin are specific for an epitope which has GalNAc asa key determinant and that this epitope is associated with cancer cell lines and not with normal non-transformed cells. Thus, the DS-OSM binding lectin produces selective apoptosis of cancer cell lines expressing the GalNAc epitope.

Example 5: Evidence of the GalNAc Epitope in Apoptosis

Endothelial cells have been shown to be rich in cell surface glycoproteins which have been proposed to carry xenogenic epitopes. The potential of PAEC to synthesize asparagine-linked oligosaccharides (N-glycans) and Ser/Thr-linked oligosaccharides (O-glycans) was investigated.

Cells to be tested were grown to confluency in 96 well microtitre plates as described in the cell culture section. The cells were fixed with 0.01% gluteraldehyde for 5 min at 4°C and washed with PBS three times. The cells were blocked with 100 µl/well of 1% BSA in PBS for 1 h at room temperature. Samples of biotinylated lectins and sugars were prepared in a dilution 96 well microtitre plate (100 µl/well) and incubated on a shaker for 2 h at room temperature. Eighty µl/well from the dilution plate were transferred to the test plate wells emptied of the blocking buffer and incubated on a shaker for 1 h at room temperature. The wells were washed with 200 µl/well of PBS three times and blocked with 100 µl/well of 1% BSA in PBS for 15 min at room temperature. The blocking solution was removed and alkaline phosphatase conjugated avidin (50 µl/well at 1 µg/ml) was incubated in the microtitre plate on a shaker for 1 h at room temperature. The cells were washed with 200 µl/well of PBS three times and 4-methylumbelliferyl phosphate (100 µl/well at 100 µg/ml) was incubated with the cells for 1 h at 37°C. The plates were scanned with a Cytofluor 3000 plate reader (ex-360 nm em-460 nm). The data were analyzed using the Microsoft Excel spreadsheet program.

Glycosyltransferase and sulfotransferase activities assembling N-glycans and O-glycans were measured by ion exchange, Sep-Pak, high voltage electrophoresis and HPLC assays as previously described (Yang J.M., et al., Glycobiology, 4:873-884, (1994); Brockhausen I, et al., Cancer Re., 51:3136-3142 (1991); Brockhausen I, et al., Eur J Biochem, 233: 607-617 (1995); Brockhausen I, et al., J Biol Chem, 264: 11211-11221 (1989)). PAEC homogenates were used as the enzyme source and prepared as follows: subconfluent PAEC were harvested and washed three times in 0.9% saline. The cell pellet was hand-homogenized in 0.25 M sucrose (1 ml sucrose per 100 million cells) and stored at -70°C.

This is the first comprehensive biosynthetic study in endothelial cells. Activities of the major known glycosyltransferases and sulfotransferases involved in the assembly of N- and O-glycans were determined in homogenates of subconfluent PAEC. It was shown that PEAC contained the enzymes synthesizing complex bi-antennary

N-glycans. Thus, GlcNAc-transferase I and II and the enzymes involved in the synthesis of polylactosamine chains were very active. GlcNAc-transferases III, IV, and V, involved in the synthesis of tri- and tetra-antennary and bisected N-glycans were barely detectable. Sialyltransferases and alpha 3 Gal-transferase terminating these chains were active. We therefore confirmed that PAEC are suitable model cells to study the role and regulation of the Gal alpha (1,3) Gal- xenogeneic epitope. In addition, PAEC cells are rich in enzymes synthesizing O-glycans as simple GalNAc-, core 1, Gal beta (1,3) GalNAc-, and core 2, GlcNAc beta (1,6)(Gal beta (1,3)) GalNAc- structures. We have especially focused on an active polypeptide GalNAc-transferase, which is the first enzyme of the O-glycan pathway and regulates the number and positions of attached O-glycan chains. This enzyme synthesizes the GalNAc-structure (Tn antigen). Our specificity studies indicated that the enzyme in PAEC cells is similar to those of bovine tissues and is minimally reactive with Ser-residues of peptides *in vitro*. The sialyl-GalNAc- and sulfo-transferases that modify O-glycan chains with core 1 and core 2 structure are present in PAEC cells at levels that are comparable to other cell types (Brockhausen I, et al., Cancer Research, 51:3136-42. (1991); Brockhausen I., The Biosynthesis of O-glycosylproteins Glycoproteins Ed. Montreuil J, Vliegenthart JFG, Schachter H, Elsevier Pub pp201-259 (1995)). Thus, the profile of GTs in PAEC is consistent with the production of xenogenic epitopes which exhibit complementarity to the isolated human serum lectins.

To demonstrate human antibody binding to GalNAc epitopes on porcine endothelial cells a cellular ELISA assay was performed. Carbohydrates were used to competitively inhibit human antibody binding. GalNAc, and GalNAc oligosaccharides were effective at inhibiting human anti-porcine antibody binding. One hundred mM GalNAc produced 74±1% inhibition of human antibody binding. Other carbohydrates such as GlcNAc, or galactosamine inhibited human antibody binding by 8-37%. DS-OSM, an asialo-mucin rich in GalNAc epitopes, was tested for its ability to inhibit human antibody binding to PAEC. Three mM of DS-OSM inhibited human antibody binding

by 88°4%. Porcine endothelial cells were examined for cell surface expression of carbohydrate epitopes with plant lectins. Lectins specific for GalNAc lectins were used in a fluorescent microtire plate assay as were lectins which had other specificities. SBA, LBA, DBA, and HP lectins (specific for GalNAc) bound to PAEC 1.5-1.7 at times control. Some lectins which were specific for Gal alpha epitopes, such as Jacalin and MPA, bound to PAECs at 1.8 times control. However, other lectins which were not specific for either GalNAc or Gal alpha epitopes, such as PWM or PNA, bound weakly to PAECs (0.9-1.1 times control) while others, such as WGA or ConA, bound PAECs at 1.6 times control. These results indicate that the GTs indicated above can produce carbohydrate epitopes that are expressed on the cell surface.

Porcine endothelial cells exposed to plant lectins specific for GalNAc epitopes underwent cell detachment. GalNAc-specific plant lectins, such as SBA, produced dose-dependent detachment of the endothelial cells. The detachment was completely inhibited by GalNAc, while other sugars did not inhibit SBA-induced detachment (Fig. 17). SBA produced detachment of PAEC to the same extent as Gal alpha-specific lectins, such as EEA, which we have shown to induce PAEC apoptosis. Likewise, GS-IA4 also showed dose-dependent detachment, and this was inhibited by GalNAc and not by other sugars. Plant lectins which did not show binding to the PAECs did not produce detachment.

Human serum proteins isolated against a DS-OSM mucin column were tested against porcine endothelial cells. The proteins were eluted from the DS-OSM column with 125 mM GalNAc or with 7.5 mM EDTA. The DS-OSM eluant was applied to a DEAE chromatography disc and eluted with a combination of EDTA and a salt gradient. The endothelial cells showed 80-90% detachment in a dose-dependent fashion when exposed to the DS-OSM/GalNAc or to DS-OSM/EDTA-eluted lectins at a concentration of 100 µg/ml. The eluant from the DS-OSM column is shown in Example 4 to be a potent apogenic stimulus in breast and lung cancer cells and, in distinction to the cancericidal agent, cyclophosphamide, to produce little or no effect

on normal control cell lines. The effect of human serum lectins can be reduced by passage of human sera through the DS-OSM column. The apogenic effect of human sera on PAEC was reduced from 39.9%±2.9% to 29.2±2.7% following DS-OSM column treatment (p=0.04). Separate evaluation of transferrin, alpha 1 antitrypsin, alpha macroglobulin, and the anti-sialyl-Tn antibody, which exhibited no apogenic effect, indicates that the apogenic effect of the GalNAc-binding lectin, both in the cancer cell lines and in the xenogenic PAEC, is attributable to the unknown protein containing the amino acid sequence given above.

The development of novel glycosyltransferase inhibitors based on structural analysis of xenogenic or cancer-specific epitopes and cognate GT activity would be anticipated to affect the susceptibility of treated cells to apoptotic injury caused by human serum lectins. Substrate analogue inhibitors of GT activities can be developed which eliminate the synthesis of apogenic epitopes based on the current knowledge of the binding sites of GT. (Brockhausen I. Glycoproteins Ed. Montreuil J, Vliegenthart JFG, Schachter H, Elsevier Pub pp201-259 (1995)). These substrate analogue inhibitors can be designed according to known GT specificities and chemically synthesized according to published methods. Modulation of surface-bound glycoconjugates in PAEC by GT inhibitors could be used to interfere with the susceptibility of cells to lectin-mediated apoptosis. Alternatively, the expression of apogenic epitopes in cancer cells may be increased to promote apoptosis in cancer cells.

In summary, this embodiment of the present invention demonstrates the presence of xenogenic lectins in human sera directed against porcine endothelial cells which produce endothelial cell apoptosis. The lectins were isolated using affinity and ion exchange chromatography and exhibited the same specificity as plant lectins directed toward the same epitope, GalNAc. Thus, the discovery of human serum lectins leads to the definition of additional xenogenic epitopes as exemplified by the case of GalNAc recognized by DS-OSM-binding lectins.

Example 6: Alternative method for isolation of DS-OSM binding lectins

Since albumin and IgG are proteins which are non-specifically co-purified along with DS-OSM-binding lectins by DS-OSM chromatography, they can be removed to increase the efficiency of DS-OSM-binding lectin purification. Sepharose Blue Fast Flow Protein G Fast Flow (Pharmacia, Uppsula, Sweden), and DS-OSM Fast Flow were equilibrated with 5 bed volumes of PBS (Sigma), and then the individual columns were linked in series. Twenty-five ml of human sera (A or AB blood type) were filtered with 22 μM filters and introduced into the Sepharose Blue column. The columns were washed with PBS at 0.3 ml/min until the absorbance at 280 nM (detected by an online spectrophotometer, Pharmacia) returned to baseline. The Sepharose Blue and Protein G columns were then separately eluted by 1 M NaCl and 0.15 M glycine pH 3 respectively. The DS-OSM column was eluted with 0.15 M EDTA, 1 M Galactose, 250 mM GalNAc and 1 M NaCl sequentially. In separate experiments, eluants from each of the columns and non-bound sera were tested for toxicity to PAEC. Eluants from the Sepharose Blue and Protein G columns had no detectable effect in the apoptosis assay. The sera depleted of albumin and/or IgG retained toxicity towards PAEC, and the highest rate of apoptosis was produced by exposure of the PAEC to the GalNAc eluant from the DS-OSM column.

Example 7: Methods to Inhibit Lectin Binding

Lectin binding could be inhibited by compounds which include peptide mimetics of the lectin ligands, carbohydrate analogues, anti-lectin antibodies, anti-antibody antibodies, and glycoprotein analogues. These compounds could be infused intravenously or immobilized on a solid matrix to deplete the lectins from blood in order to attenuate xenograft rejection.

For the production of antibodies against purified lectins, about 100 μg of purified lectin mixed with Freund's adjuvant could be injected into rabbits. The production of

antibodies could be measured by an ELISA assay using purified lectin. The injections could be repeated after six weeks until a high titre was achieved. These monoclonal antibodies could be prepared by conventional procedures, generally following the methods of Kohlers and Milstein (Nature, 256:495-497 (1975); Eur. J. Immunol. 6: 511-519 (1976)). According to these methods, tissue culture adapted mouse myeloma cells are fused to antibody producing cells from immunized mice to obtain the hybrid cells that produce large amounts of a single antibody molecule. In general the antibody producing cells are prepared by immunizing an animal (e.g. mouse, rat, rabbit, sheep, horse, or cow) with an antigen. The immunization schedule and the concentration of the antigen in suspension is such as to provide useful quantities of suitably primed antibody producing cells. These antibody producing cells can be either spleen cells, thymocytes, lymph node cells, and/or peripheral blood lymphocytes.

The antibody producing cells are then fused with myeloma cells or cell lines originating from various animals such as mice, rats, rabbits, and humans using a suitable fusion promoter. Many mouse myeloma cell lines are known and available generally from members of the academic community and various depositories, such as the American Type Culture Collection, Rockville, MD. The myeloma cell line used should preferably be medium sensitive so that unfused myeloma cells will not survive in a selective media, while hybrids will survive. The cell line most commonly used is an 8-azaguanine resistant cell line, which lacks the enzyme hypoxanthine-guanine-phosphoribosyl-transferase and therefore will not be supported by HAT (hypoxanthine-aminopterin-thymidine) medium. In general, the cell line is also preferably a "non-secretor" type, in that it does not produce any antibody. The preferred fusion promoter is polyethyleneglycol having an average molecular weight on the range of 1,000 to about 4,000 kD. Other fusion promoters such as polyvinylalcohol, a virus or an electrical field can also be used.

The immortalized cells (hybridoma) must then be screened for those which secrete antibody of the correct specificity. The initial screening is generally carried out using

an ELISA. Specifically, the hybridoma culture supernatants are added to microtitre plates which have been previously coated with the antigen, in this case the individual lectin. A bound specific antibody from the culture supernatants can be detected using a labelled second antibody, for example, goat antimouse IgG labelled with peroxidase (commercially available). Cultures that are positive against the antigen are then subjected to cloning by the limiting dilution method. These polyclonal or monoclonal antibodies then can be used to inhibit or reduce xenograft rejection.

The production of scFv, Fab, F(ab')2, diabodies, and minibodies are predicated on the cloning and production of antibody variable domains. These methods and reagents are readily available through commercial vendors such as Pharmacia Biotech and Cambridge Antibody Technology Ltd., Cambridge, U.K. The general method involves isolating mRNA from murine hybridoma cells (produced as noted above) and amplified by PCR using a light and heavy chain primer mix specific for the variable region of each. The purified heavy and light chain DNA are annealed together with DNA sequences for a Gly4Ser linker to produce a scFv DNA fragment. Sfi I and Not I restriction sites are added to the 5' and 3' ends, respectively, by PCR amplication using those restriction site primers. The scFv gene fragment is then purified, digested with Sfi I and Not I, purifed, and ligated into the pCANTAB 5 E phagemid. The phage can be used to infect E. coli TG1 and the M13KO7 helper phage can be used to rescue the phagemid and antibody scFv gene. The rescued phage expresses the scFv antibody fragments and can be used in screening. In this case, the phages can be panned against each individual lectin coated on plates and then washed several times with a buffer. The phages that bind the lectin could be eluted with 0.15 M glycine pH 2.5 to remove the phages that bind the lectins. The eluted phages are used to reinfect E. coli TG1. The colonies are transferred into microtitre plates and rescued with the KO7 helper phage. The phages can then be screened in an ELISA with immobilized lectin and HRP-conjugated anti-M13 antibody to detect phage coat protein. The positively identified phages are used to infect E. coli HB2151 cells, which will recognize an amber translational stop codon encoded by the pCANTAB 5 E vector. The resultant soluble scFv antibodies could be

screened in the detachment assay in conjunction with the lectins as noted above to demonstrate protection against human sera. (Chiswell D. J., et al., Trends In Biotechnology, 10:80-4(1992). Clackson T. et al., Nature, 352: 624-8 (1991); Marks J. D., et al., Journal of Molecular Biology, 222: 581-97 (1991); McCafferty J., et al., Nature, 348: 552-4 (1990); Winter G., and Milstein C. Nature 349: 293-9 (1991)).

The antigen-combining site of antibodies is comprised of the six loops that are formed by the complementarity determining regions of antibody heavy and light chains. Sometimes the epitope formed by the antigen combining site can be mimicked by small peptides. A peptide binding to the ligand binding site of a lectin may disrupt its ability to bind its cognate ligand. The interference of the lectin binding can lead to the inhibition of apoptosis in those cells susceptible to lectin-mediated apoptosis. These peptides can be obtained by screening commercial peptide libraries in the detachment assay (Chiron Mimotopes, San Diego, CA). D-amino acids, unnatural amino acids and derivatized amino acids could be incorporated into 8mer to 20mer peptide libraries based on multipin synthesis techniques. In this mimotope approach, peptide mixtures are synthesized on pins using a starting templet of *-*-*-D1-D2-*-*-*, where D1 and D2 are two defined amino acids and * represents randomly incorporated amino acids resulting from the coupling of the mixture of activated amino acids. The cleaved peptides can be screened for their ability to inhibit PAEC apoptosis by transferring them to microtire plates containing a toxic dose of lectins and then testing them against PAEC in the detachment apoptosis assay. This establishes the identity of D1 and D2 (designated in this example as X and Y) and allows the production of the compounds for the next step in this iterative process: *-*-*-X-Y-D-*-*. The end result of this process will generate peptide candidates that have the capacity to inhibit lectin-mediated apoptosis, since the screening of the peptides was based on inhibiting apoptosis. The 8mer generated from this initial screening could be extended in both the N and C terminal directions using the same scheme to generate larger peptides that may have greater efficacy than the original peptide with regard to the inhibition of lectin-mediated PAEC apoptosis (Burrows S. R., et al., European Journal of Immunology, 22:191-5 (1992); Gallop M. A., et al.,

Journal of Medicinal Chemistry, 37:1233-51 (1994); Geysen H. M., et al., Proceedings of the National Academy of Sciences of the United States of America, 81:3998-4002 (1984); Maeji N. J., et al., Peptide Research, 4:142-6 (1991); Maeji N. J., et al., Journal of Immunological Methods, 146:83-90 (1992); Maeji N. J., et al., Peptide Research, 8:33-8 (1995); Reece J. C., et al., Journal of Immunology, 151:6175-84 (1993); Valerio R. M., et al., International Journal of Peptide & Protein Research, 42:1-9 (1993); Wang J. X., et al., International Journal of Peptide & Protein Research, 42:384-91 (1993); Wang J. X., et al., International Journal of Peptide & Protein Research, 42:392-9 (1993); Weiner A. J., et al., Proceedings of the National Academy of Sciences of the United States of America, 89:3468-72 (1992)). Although we describe only one scheme of combinatorial peptide synthesis there are many other examples extant in the literature which can be used in conjunction with the detachment assay to generate compounds that can effectively inhibit lectin-mediated apoptosis.

The production of peptide libraries is not limited to those that are chemically synthesized but can include those constructed genetically by expressing the genes that encode those amino acids (Smith G. P. and Scott, J K. Methods in Enzymology, 217:228-57 (1993). Yu J. and Smith, G P., Methods in Enzymology, 267:3-27 (1996)). In this method, synthetic degenerate oligonucleotide inserts are constructed in the format X(NNK)nY, where X and Y correspond to restriction sites and NNK represent a codon where N is a position synthesized using equimolar mixture of dA, dC, dG, and T, but K is formed by an equal mixture of dG and T; n determines the number of codons. If X and Y correspond to Sfi I and Not I restriction sites, then the same methods for the expression phage antibodies could be used (as described above) to express the peptides. The phages expressing the peptides could be panned against immobolized lectins and washed under low stringency conditions. The DNA isolated from the eluted phage forms the template for PCR amplification. The DNA could be mutagenized to obtain clones with a range of affinities, some of which will have greater effect at inhibiting lectin-mediated apoptosis. One method of mutagenesis is the use of error prone PCR to amplify the DNA extracted from the

eluted phages. Inosine 5'-triphosphate could be used to spike the reaction mixture to increase the rate of base substitutions by DNA polymerase during the PCR process (Gram H., et al., Proceedings of the National Academy of Sciences of the United States of America, 89:3576-80 (1992)). The phages could be screened against the lectins under higher stringency conditions and those phages could be used to produce soluble peptide. The phages that produce the most efficacious peptides for inhibiting lectin-mediated apoptosis in the detachment apoptosis assay assay could undergo further rounds of mutagenesis and selection.

In order to interfere with lectin binding to reduce apoptosis inhibitors, could be derived from the geometry of the carbohydrate binding site of human serum lectins. Recombinant lectin could be used to raise crystals for x-ray crystallography, with and without carbohydrate ligand. The information gained from these studies would indicate the precise dimensions of the binding site and the spatial contacts between the carbohydrate ligand and amino acids of the lectin. Site-directed mutagenesis experiments aimed at mutating these specific amino acids would confirm their role in ligand binding. Based on these studies, synthetic carbohydrates could be synthesized that are expected to form better contact points for the carbohydrate binding site of the lectin. This would produce lead compounds which can be optimized through high throughput screening of chemically related compounds (not necessarily carbohydrates) using the apoptosis detachment assay.

The teachings embodied in this example can lead to the production of compounds to inhibit apoptosis based on screening large libraries, either biologic or chemically synthesized, in the detachment apoptosis assay. The specific example is that of compounds to inhibit human serum lectins that mediate PAEC apoptosis which may be useful to prevent xenograft rejection. These techniques would apply to other apogens and cell types.

Example 8: Identification of Apogens Using the Apoptosis Detachment Assay

The apoptosis detachment assay can be used to identify apogens derived from large libraries of compounds. In one example the production of apogens could be predicated on the structure of apogenic human serum lectins identified in present invention. Specifically, the actions of the described lectins could be mimicked by anti-idiotypic antibodies directed against antibodies that antagonize the actions of the lectins described in Example 7 (i.e., polyclonal antibodies, monoclonal antibodies, or coliform antibodies derived from phage).

The antigen-combining site of an antibody is comprised of unique determinants, referred to as idiotopes, which can be recognized by other antibodies. These anti-idiotypic antibodies are the mirror image of the antigen-combining site of the first antibody and topologically resemble the inital antigen, in this case the lectin. The anti-idiotypic antibodies can be produced with either isolated antibodies or phage antibodies. Anti-idiotypic antibodies against human anti-lectin antibodies could be produced by polyethylene glycol mediated cell fusion between Sp2/0 myeloma cells and immunocytes obtained from the spleen of Balb/c mice immunized with either 50-75 μg of purified anti-lectin polyclonal antibodies or monoclonal antibodies (Young D S F, et. al. Inhibition of human xenogenic or allogenic antibodies to reduce xenograt or allograft rejection in human recipients. 1996 US PTO 08-867283WO). Primary injections could be given subcutaneously with the immunogen emulsified in Freund's complete adjuvant (FCA), followed by two boosts intraperitoneally (i.p.). The first boost could be administered on day 21 with Freund's incomplete adjuvant (FIA) and the second boost at day 45 with adjuvant-free immunogen. The mice could be sacrificed three days after the second boost to isolate splenocytes for cell fusion and then hybridomas could be cloned out according to the method of Takahashi (Takahashi, M., et al., Journal of Immunological Methods, 96(2):247-53 (1987)). Alternatively, phage displayed antibodies could be used for innoculation since phages are highly immunogenic

(Galfre G., et al., Methods in Enzymology, 267:109-115 (1996)). In this case, 1011 phage (approximately 1.3 μg of protein for all innoculations) in FCA are adminstered i.p. followed by the first boost on day 21 with FIA and again on day 42. The mice could be sacrificed 10 days later and the splenocytes could be isolated for cell fusion to produce hybridomas as noted above. Regardless of the immunogen, the antibody genes can also be rescued using the phage display methods outlined in Example 7. In a separate method, antibodies directed against the cells of interest could be produced directly. Instead of using anti-lectin antibodies for immunization, cells grown to confluency in T-flasks could be harvested into a single cell suspension and fixed in 0.1% gluteraldehyde in PBS, pH 7.4. The fixed cells could then be washed four times in a centrifuge with PBS. After the last wash, the cell pellet could be resuspended in sterile PBS to a cell density of $2 \times 10^8$ cells/ml. Balb/c mice could be given the primary injection with the fixed cells emulsified in FCA subcutaneously. At week 3 a boost injection could be given with FIA i.p., and at week 9 a second boost could be given without adjuvant via i.p. For all injections, $1 \times 10^7$ fixed cells could be used per mouse. Three days after the last injection, the mice could be sacrificed to obtain splenocytes for cell fusion and production of hybridomas. The hybridoma supernatants derived could be screened in the detachment assay against the cells of choice (e.g. T47D cells in the case of breast cancer or Calu-3 in the case of lung cancer) to ascertain the effectiveness of these anti-idiotypic antibodies to induce apoptosis. The phage derived from the spleens of the immunized mice could be screened by panning against fixed cultured cells and propagated as described in Example 7. Soluble scFv derived from microtitre plates seeded with plaques of HB2151 cells infected by positive binders could be assayed in the detachment assay against the cells of interest, as noted above. The use of cells to directly innoculate the mice would not guarantee that the resultant antibodies would be related to lectins, but rather this method leads to random generation of antibodies that directly induce apoptosis of the target cells.

A modification of the method for the production of phage display anti-idiotypic antibodies is the use of gene sequences derived from the lectin genes. These fragments

could either be random fragments or based on tryptic digests of the lectin that are apogenic in the apoptosis detachment assay. The peptide fragments could be sequenced, and based on the molecular weight, the length of DNA could be estimated for the design of flanking primers. Random mutations could be introduced into these gene segments at the time of amplification by the use of error prone PCR. The fragments could be ligated into the pCANTAB 5 E phagemid vectors after the addition of Sfi I and Not I restriction sites to the 5' and 3' ends, repectively, using appropriate restriction site primers by PCR. The method to express the lectin fragments and their derivatives could follow the methods of phage display of antibodies as described in Example 7. The screening of phages that carry peptides of interest (i.e., peptides that bind the cells of interest and ultimately contribute to the apoptosis of those cells) could follow the methods described above for screening phages conveying anti-idiotypic antibodies that mediate cellular apoptosis. We recognize the possibility exists that apogenic peptides derived using this method might not retain the original lectin specificity, but this possibility can be formally tested by carbohydrate-binding studies.

Peptide libraries (both chemically synthesized and genetically based) described in Example 7 could be screened in the detachment apoptosis assay to discover apogens. Synthetic peptides could be solubilized and tested in the detachment assay against the cells of interest. Positive hits, however derived, could be further characterized by testing against normal human cells such as HAEC or HPAEC to determine the relative toxicity of the peptide. The use of the detachment apoptosis assay to discover novel apogens is not limited to peptide compounds but can also include inorganic as well as other biological libraries. There are several examples of commercially available small-molecule libraries, including the DIVERSet collection of small molecues (ChemBridge Corporation, Northbrook, IL and Chemical Design, Cromwell Park, UK) and the Optiverse library from Panlabs (Panlabs, Bothell, WA).

Example 9: Identification of Lectin Receptors:

Once large amounts of recombinant lectins are expressed, the corresponding cell surface receptors of PAEC could be isolated in order to develop methods to make these cells more resistant to the action of apogenic lectins. Prevention of human lectin binding to PAEC may be accomplished by infusion of chemically synthesized, small haptenic glycopeptides or by down regulation of cell surface glycoprotein expression. In cancer cells, an enhancement of lectin action may be achieved by overexpression of the cell surface glycoproteins which bear the lectin ligand or by overexpression ofglycosyltransferases directly involved in the synthesis of the carbohydrate ligands. A number of proteins are expected to have similar receptor properties which can be targeted by these techniques.

Isolation of the cell surface receptors for human lectins on PAEC could be undertaken from detergent-solubilized PAEC. Recombinant lectin could be coupled to a resin and used as an affinity column to bind solubilized cell surface receptors. The receptors could be eluted from the column with competing carbohydrates, and the procedure could be repeated several times. Additional chromatographic steps could be undertaken to obtain pure receptor species. The peptide moiety could be identified by preparing tryptic peptides followed by gas phase amino acid sequencing. Laser desorption mass spectrometry and other methods could be used to identify the molecular weight of the glycoprotein and the number and attachment sites of carbohydrate chains.

The isolation of the cell surface lectin receptor from cancer cells could be accomplished using similar methods. For example, epithelial cancer cells such as T47D cells are known to display large amounts of cell surface mucin MUC1 carrying GalNAc residues. The isolation of MUC1 receptors therefore could be achieved by lectin affinity columns followed by anti-MUC1 antibody affinity chromatography.

The identification of lectin receptors could to lead to strategies designed to promote or

inhibit lectin-mediated apoptosis and to the rational design of lectin analogues. In addition, characterization of lectin receptors would be expected to yield information regarding receptor-mediated apoptotic signalling.

The present invention has been described with regard to preferred embodiments. However, it will be obvious to persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as described in the following claims.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. An isolated human serum apogen and variants thereof, capable of inducing apoptosis.

2. The apogen of Claim 1, wherein the apogen is a heat-labile human apogen.

3. The apogen of Claim 2, wherein the apogen is a human serum lectin.

4. The apogen of Claim 3, wherein the human serum lectin induces apoptosis in cells selected from the group consisting of xenogenic cells, transformed cells and cancer cells.

5. The apogen of Claim 4, wherein the xenogenic cells are selected from the group consisting of sheep cells, mouse cells, non-human primate cells, kangaroo cells, ostrich cells and porcine cells.

6. The apogen of Claim 5, wherein said human serum lectin is selected from the group consisting of a Gal alpha (papGal, melibiose) binding protein, a GalNAc (DS-OSM) binding protein, a GlcNAc binding protein, a fucose binding protein, a mannose binding protein and a lactose binding protein.

7. The apogen of Claim 6, wherein said human serum lectin is selected from the group consisting of a Gal alpha (papGal) binding protein, a GalNAc binding protein and a GlcNAc binding protein.

8. The apogen of Claim 7, wherein said human serum lectin is a Gal alpha (papGal) binding protein and the xenogenic cell is porcine endothelial cells.

9. The apogen of Claim 8, wherein said Gal alpha (papGal) binding protein is selected from the group consisting of a Gal alpha (papGal) binding protein with a molecular weight of 60 kD, a Gal alpha (papGal) binding protein with a molecular weight of 32 kD, a Gal alpha (papGal) binding protein with a molecular weight of 73 kD and a Gal alpha (papGal) binding protein with a molecular weight of 22 kD.

10. The apogen of Claim 8, wherein said Gal alpha (papGal) binding protein is a Gal alpha (papGal) binding protein with a molecular weight of 73 kD.

11. The apogen of Claim 7, wherein said human serum lectin is a GalNAc binding protein and said human serum lectin induces apoptosis in cells selected from the group consisting of xenogenic cells, transformed cells and cancer cells.

12. The apogen of Claim 11, wherein said cancer cells are selected from the group consisting of breast cancer cells, colon cancer cells, neural cancer cells, pancreatic cancer cells, lung adenocarcinoma cells, prostate cancer cells, sarcoma cancer cells, testicular cancer cells, and metastatic cells derived from the above cancers.

13. The apogen of Claim 11, wherein the xenogenic cells are selected from the group consisting of sheep cells, mouse cells, non-human primate cells, kangaroo cells, ostrich cells and porcine cells.

14. The apogen of Claim 13, wherein the GalNAc binding protein comprises a peptide having a partial amino acid sequence of I/LKSDALKSVSQGP.

15. The apogen of Claim 1, wherein the variants include a compound that is structurally similar to the human serum apogen, wherein said compound does not impair binding or apogenic capacity.

16. The apogen of Claim 15, wherein the compounds are selected from the group consisting of peptide mimetics, inorganics, antibodies, antibody fragments and phage

display proteins.

17. A method of inducing apoptosis of cancer cells, comprising the steps of contacting said cancer cells with an apogen of Claim 1, wherein said cancer cells have abnormal glycosylation recognized by the apogen.

18. The method of Claim 17, wherein said human serum lectin is a GalNAc binding protein.

19. The method of Claim 18, wherein the GalNAc binding protein has a partial amino acid sequence of I/LKSDALKSVSQGP.

20. A method of Claim 19, wherein the cancer cells are selected from the group consisting of breast cancer cells, colon cancer cells, neural cancer cells, pancreatic cancer cells, lung adenocarcinoma cells, prostate cancer cells, sarcoma cancer cells, testicular cancer cells, and metastatic cells derived from the above cancers.

21. A method for the detection and quantification of cellular apoptosis using a fluorescence plate reader assay based on the uptake of supervital dyes by viable adherent cells, comprising the steps of:

adding a test apogen to cells grown to confluency in a cell culture plate;
washing the plate to remove detached cells;
adding a metabolic live dye to label adherent cells; and
quantifying the adherent cells in a fluorescent plate reader.

22. The method of Claim 21, wherein the metabolic live dye is selected from the group consisting of: Calcein AM, Carboxycalcein, Fluorescein diacetate, BCECF, Carboxynaphthofluorescein diacetate.

23. The method of Claim 22, wherein the method is used to screen large numbers of test apogens for potential new drugs.

24. A method of blocking an apogen of Claim 1, to inhibit or reduce apoptosis, wherein said method comprises adding to the apogen a compound which inhibits the action of said apogen.

25. The method of Claim 24, wherein said apogen is a human serum lectin.

26. The method of Claim 25, wherein said compound is selected from the group consisting of peptides, carbohydrate analogs, anti-lectin antibodies, anti-lectin antibody fragments, anti-antibody antibodies and other inorganic compounds that inhibit lectin binding.

27. The method of Claim 26, wherein said compound is used to inhibit or reduce xenograft rejection.

28. The method of Claim 27, wherein the method to inhibit or reduce xenograft rejection comprises:

administering to a recipient animal, in a pharmaceutically acceptable carrier, an effective amount of said compound, which will bind to a human serum lectin of the recipient animal to inhibit or reduce xenograft rejection mediated by human serum lectin induced apoptosis.

29. A compound for inhibiting the binding of an apogen of Claim 1, and thus for inhibiting apoptosis.

30. The compound of Claim 29, wherein said apogen is a human serum lectin.

31. The compound of Claim 30, wherein the said compound is selected from the group consisting of peptides, carbohydrate analogs, anti-lectin antibodies, anti-lectin antibody fragments, anti-antibody antibodies and other inorganic compounds that inhibit lectin binding.

32. An isolated DNA sequence encoding an apogen of Claim 1.

33. The DNA sequence of Claim 32, encoding the amino acid sequence I/LKSDALKSVSQGP.

34. A method of monitoring the serum levels of an apogen of Claim 1, wherein said method comprises providing an antibody to the apogen and using said antibody in a monitoring test.

35. An epitope defined by an apogen of Claim 1.

36. The epitope of Claim 35, wherein said epitope is selected from the group consisting of Gal alpha, GlcNAc and GalNAc.

37. A method for the diagnosis and monitoring of bound or secreted, cancer associated carbohydrate epitopes, wherein said method comprises providing an antibody to an apogen of Claim 1 and using said antibody in a monitoring test.

38. A method of targeting drugs to an epitope of Claim 35, wherein said method comprises physically linking said drug to a corresponding lectin of said epitope.

39. A method for modifying an epitope of Claim 35, by enzymatic treatment or by Glycosyl-transferase inhibitors to reduce the binding of a corresponding lectin to said epitope.

40. A method of modifying an epitope of Claim 35, by using drugs that effect Glycosyl-transferase activity

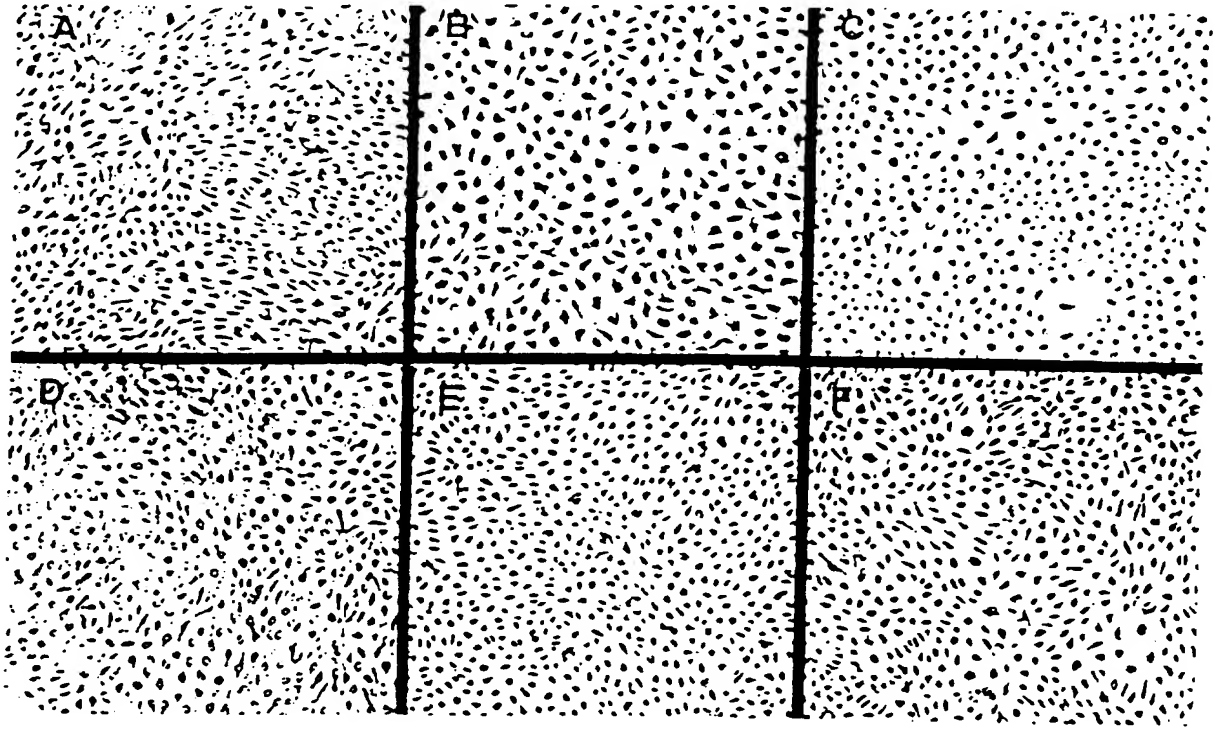



FIGURE 1

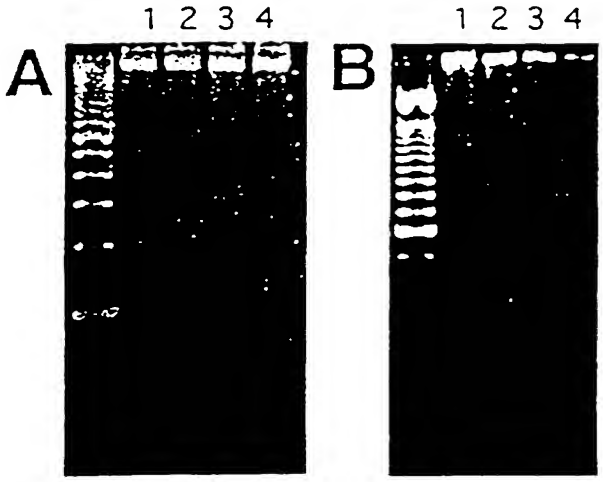



FIGURE 2

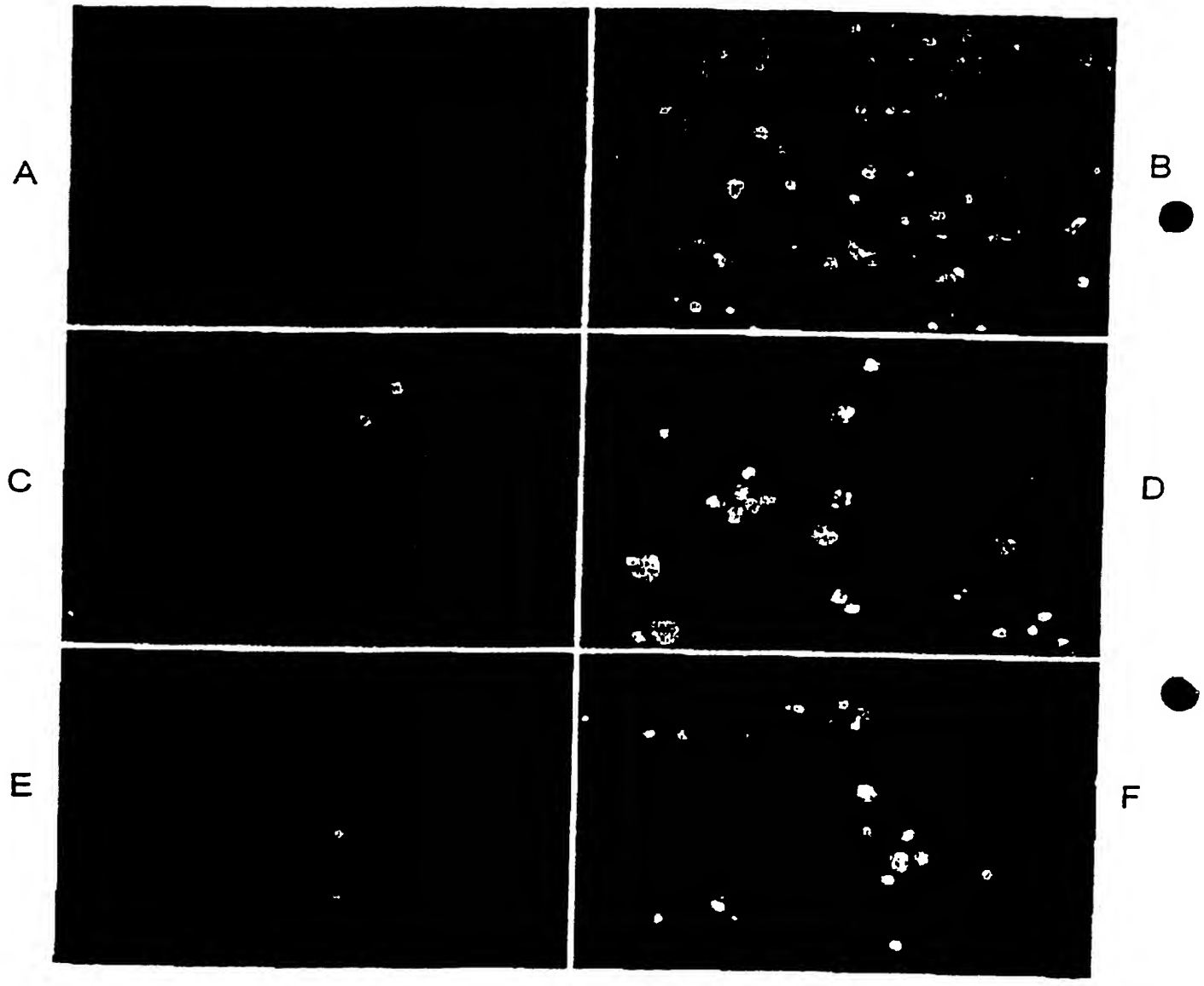



FIGURE 3

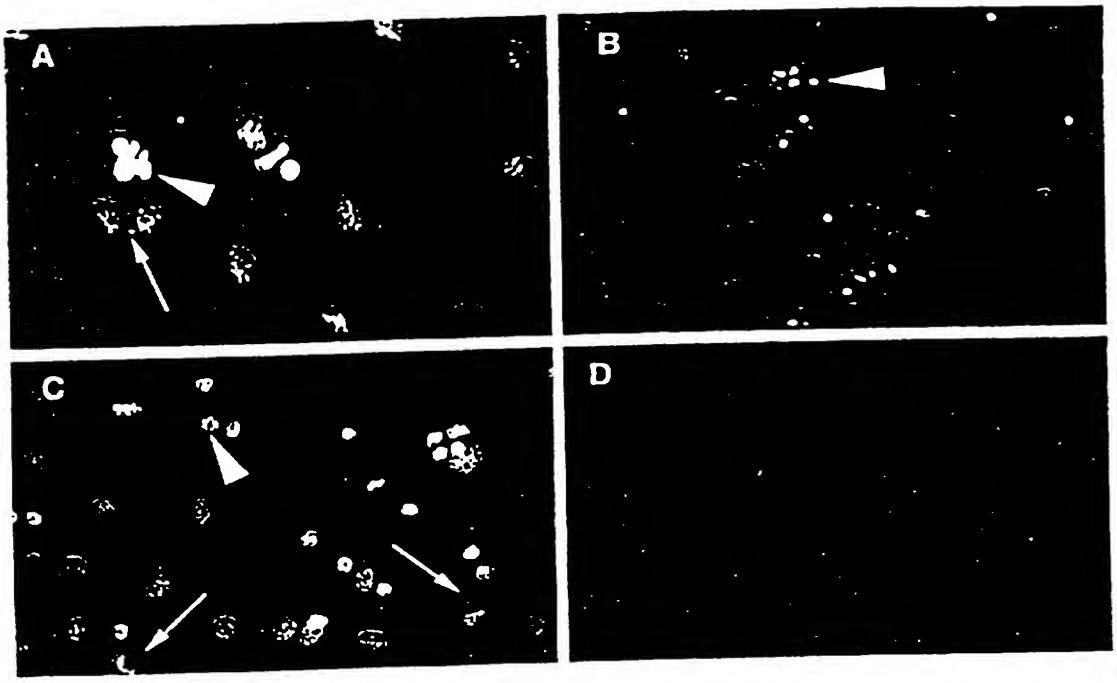



FIGURE 4

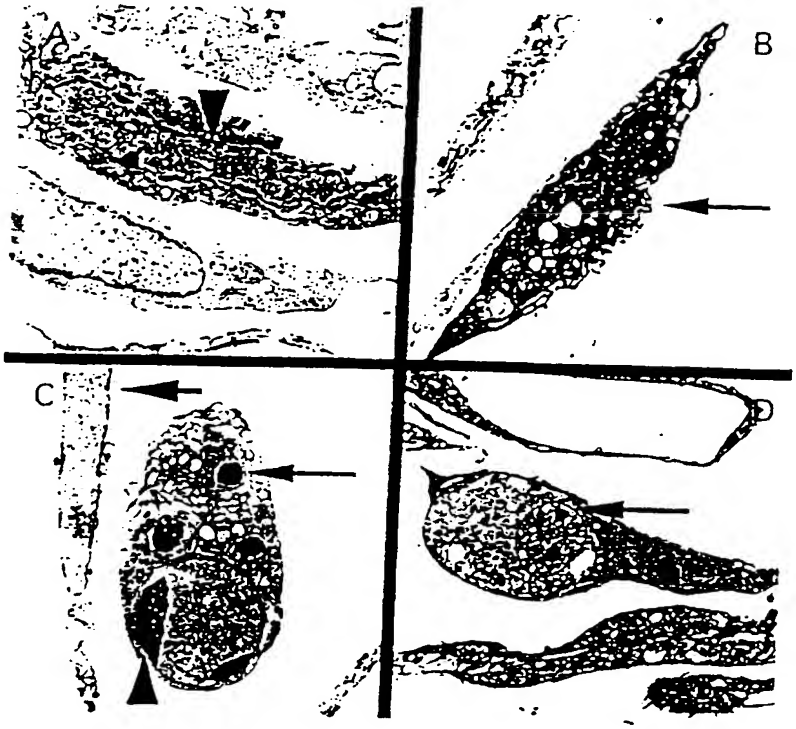



FIGURE 5

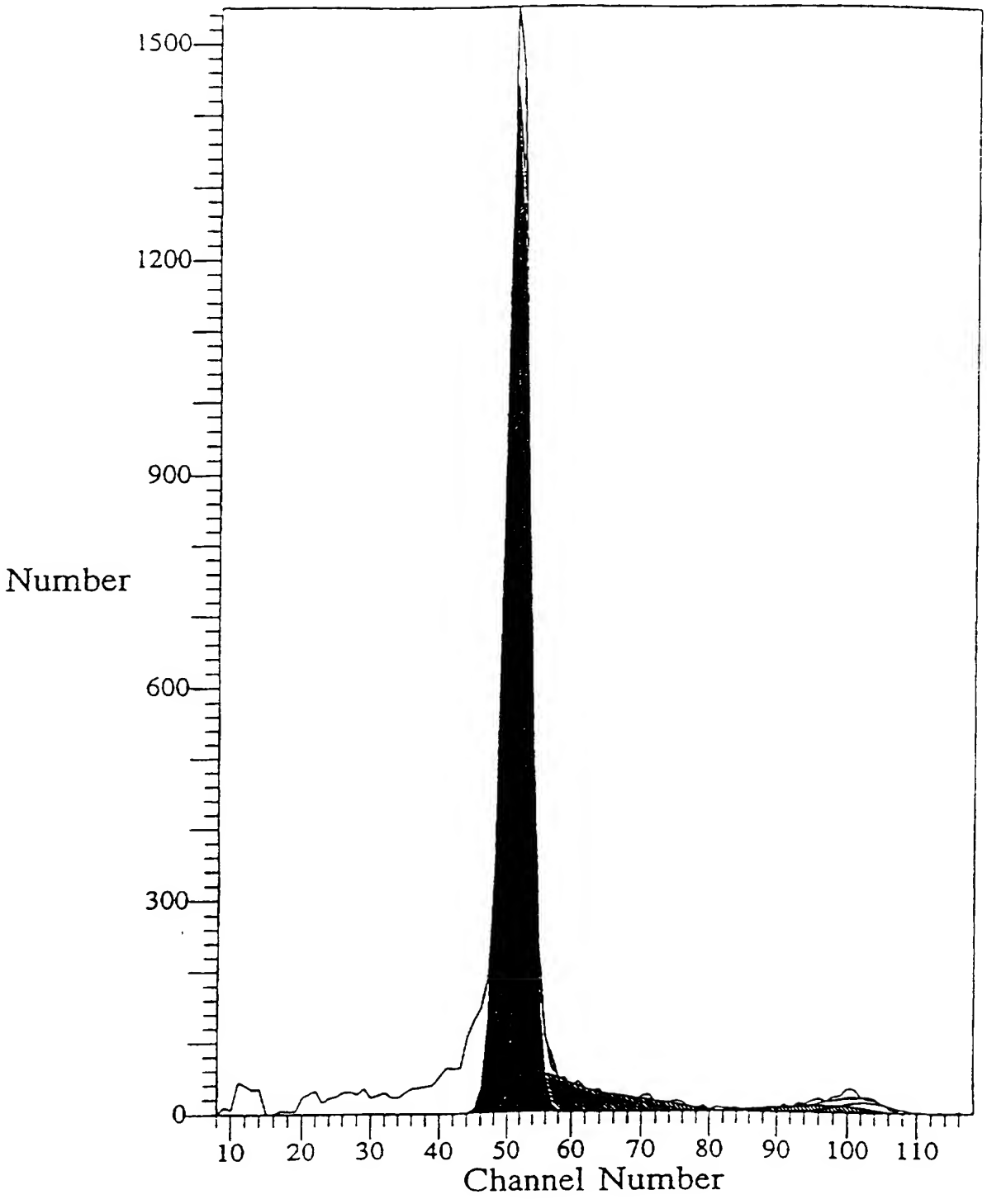
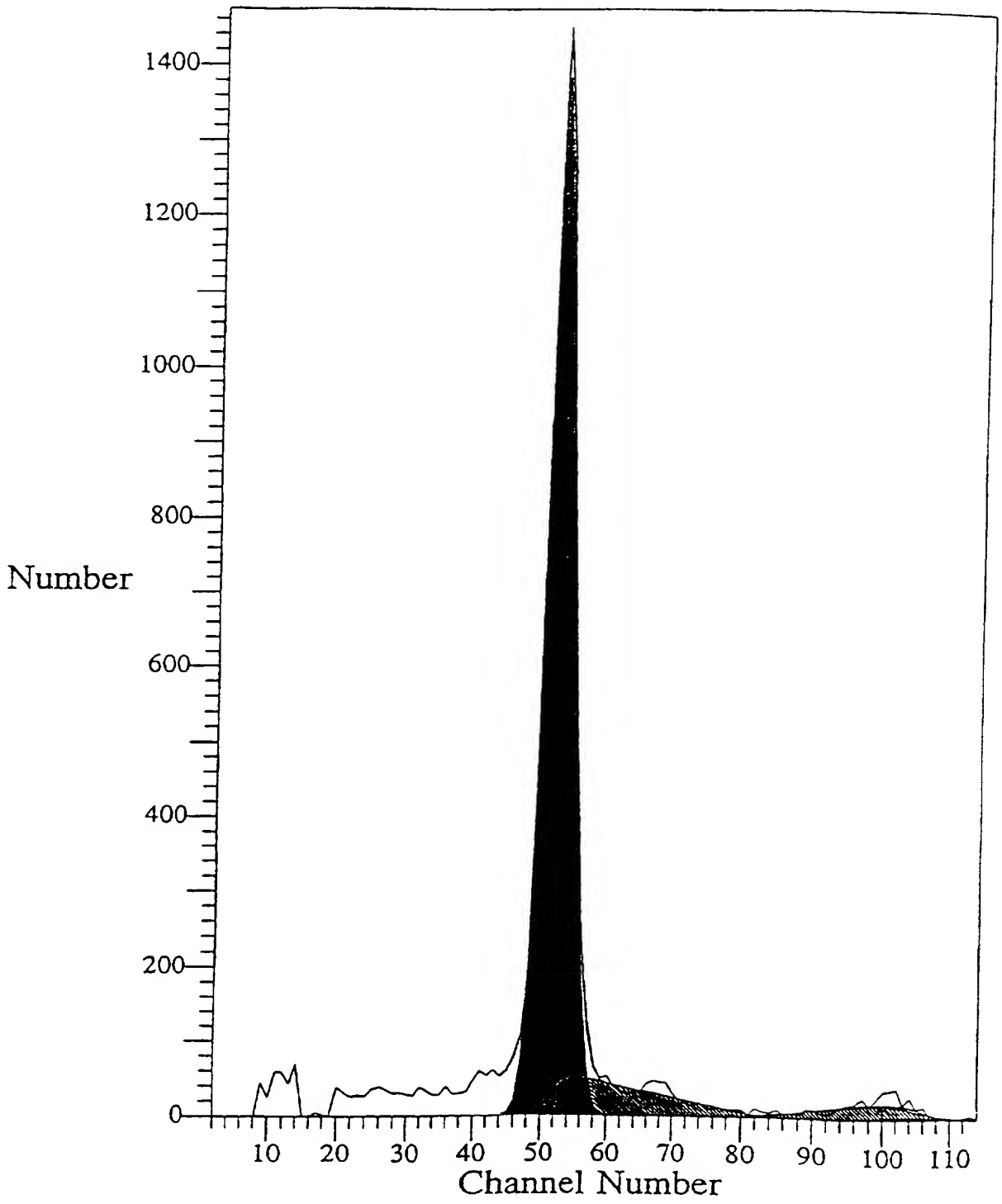

1500
1200
900
600
300
0
Number
10
20
30
40
50
60
70
80
90
100
110
Channel Number

FIGURE 6A


Number
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1200
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800
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20
30
40
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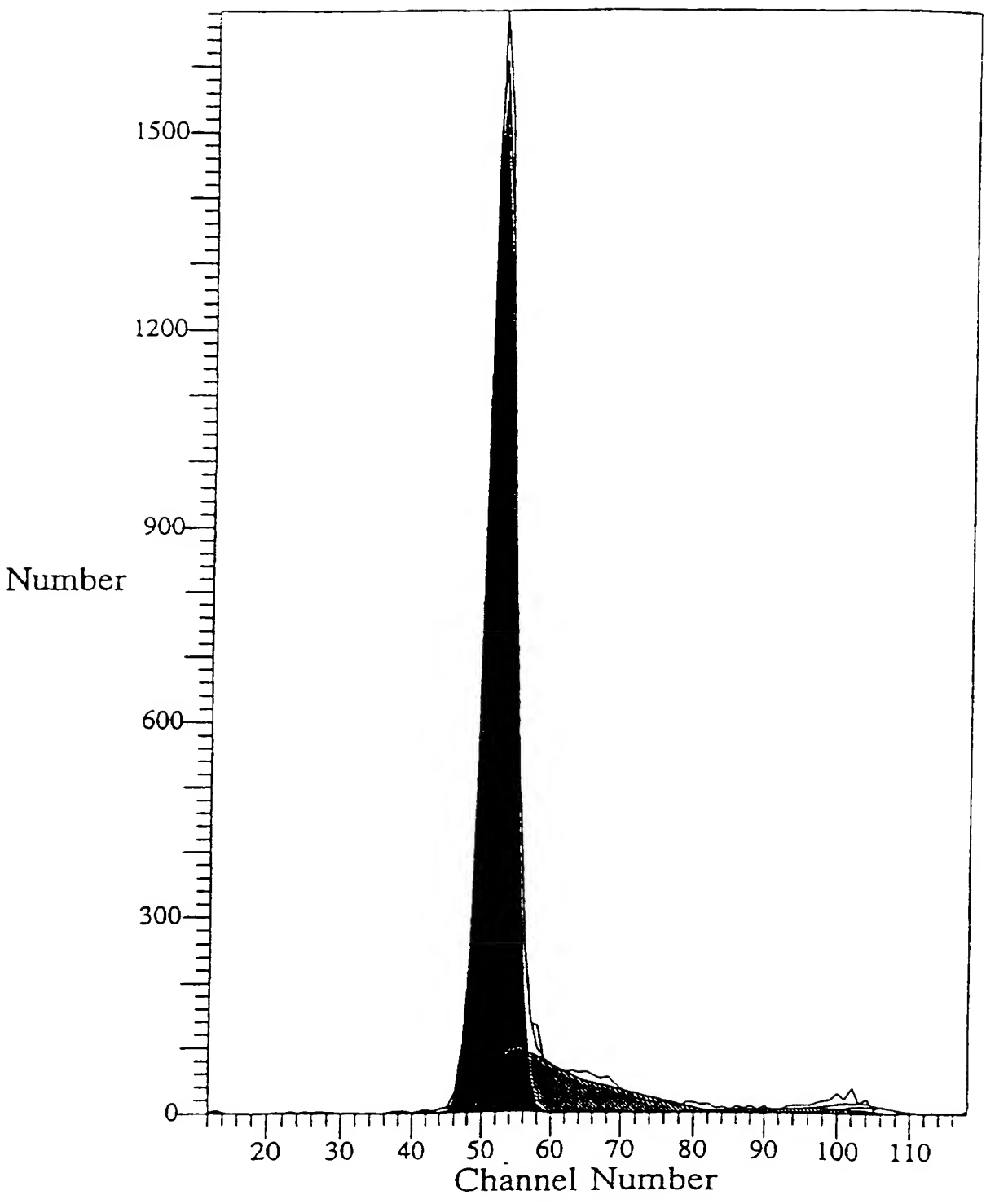
110
Channel Number

FIGURE 6B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80
90
100
110
Channel Number

FIGURE 6C

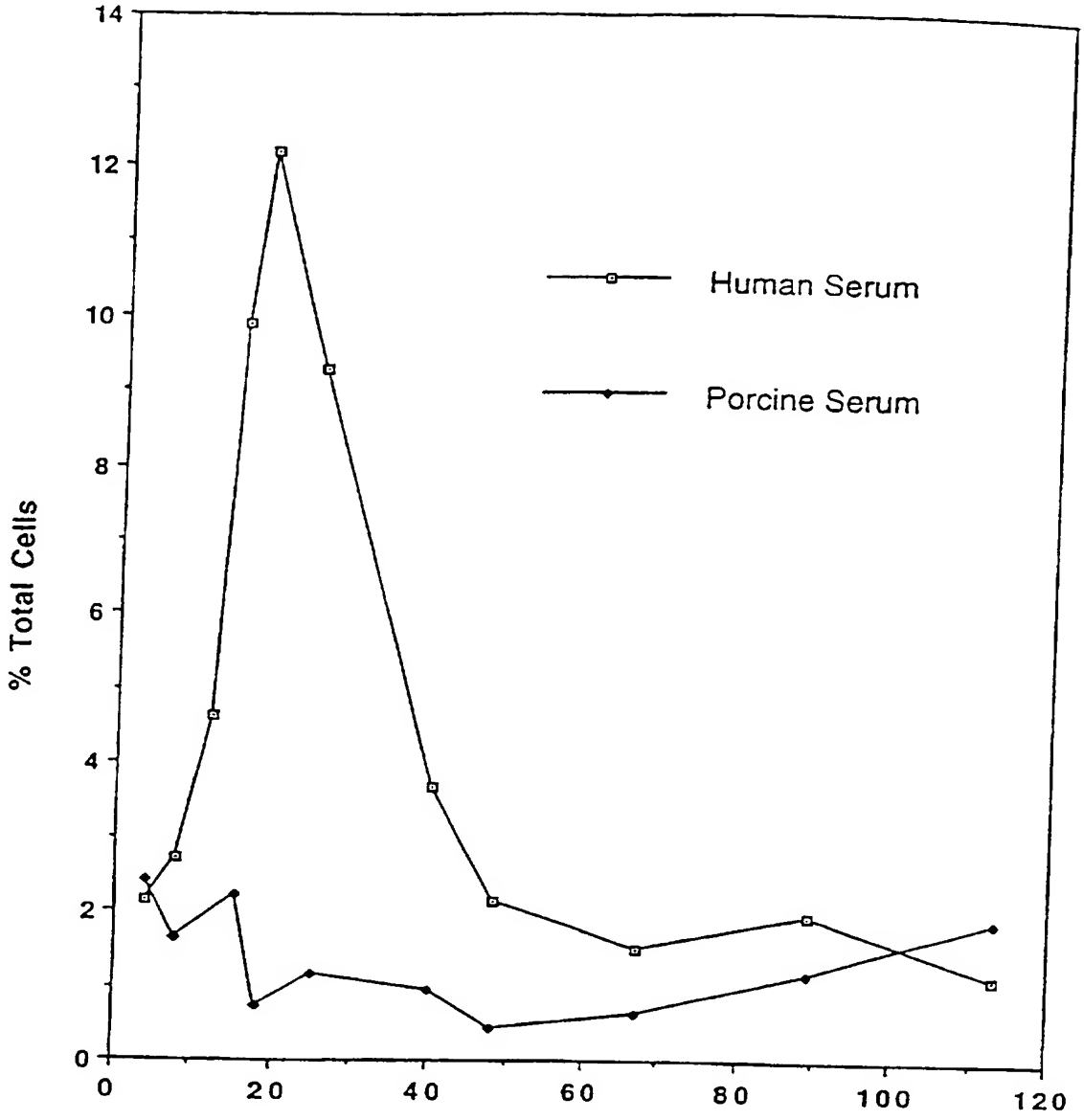
14
12
10
8
6
4
2
0
% Total Cells
Human Serum
Porcine Serum
0
20
40
60
80
100
120
Time (hours)

FIGURE 6D

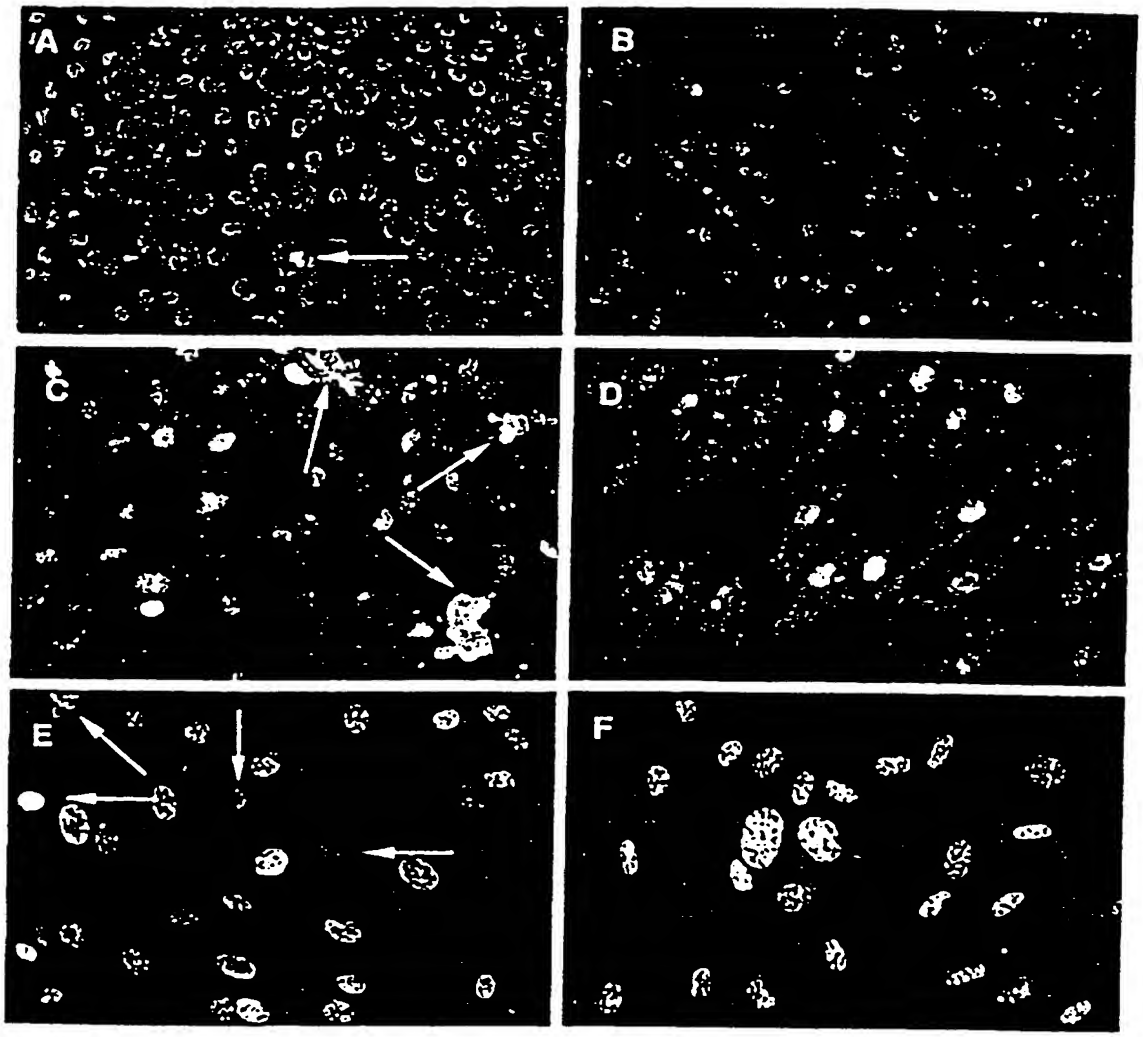


FIGURE 7

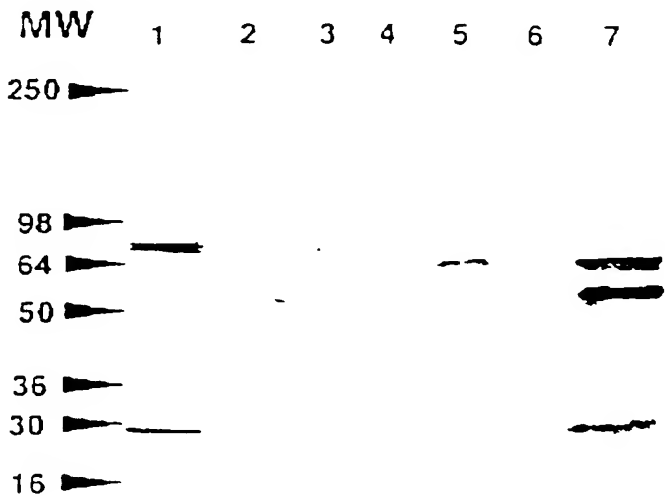


FIGURE 8

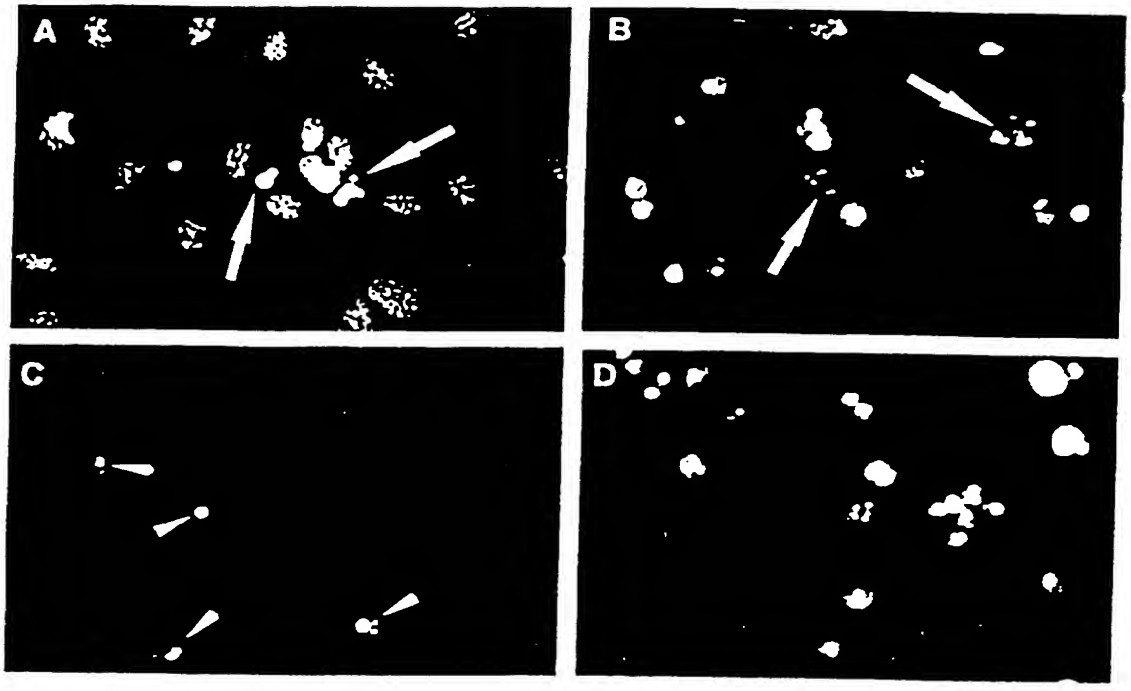


FIGURE 9

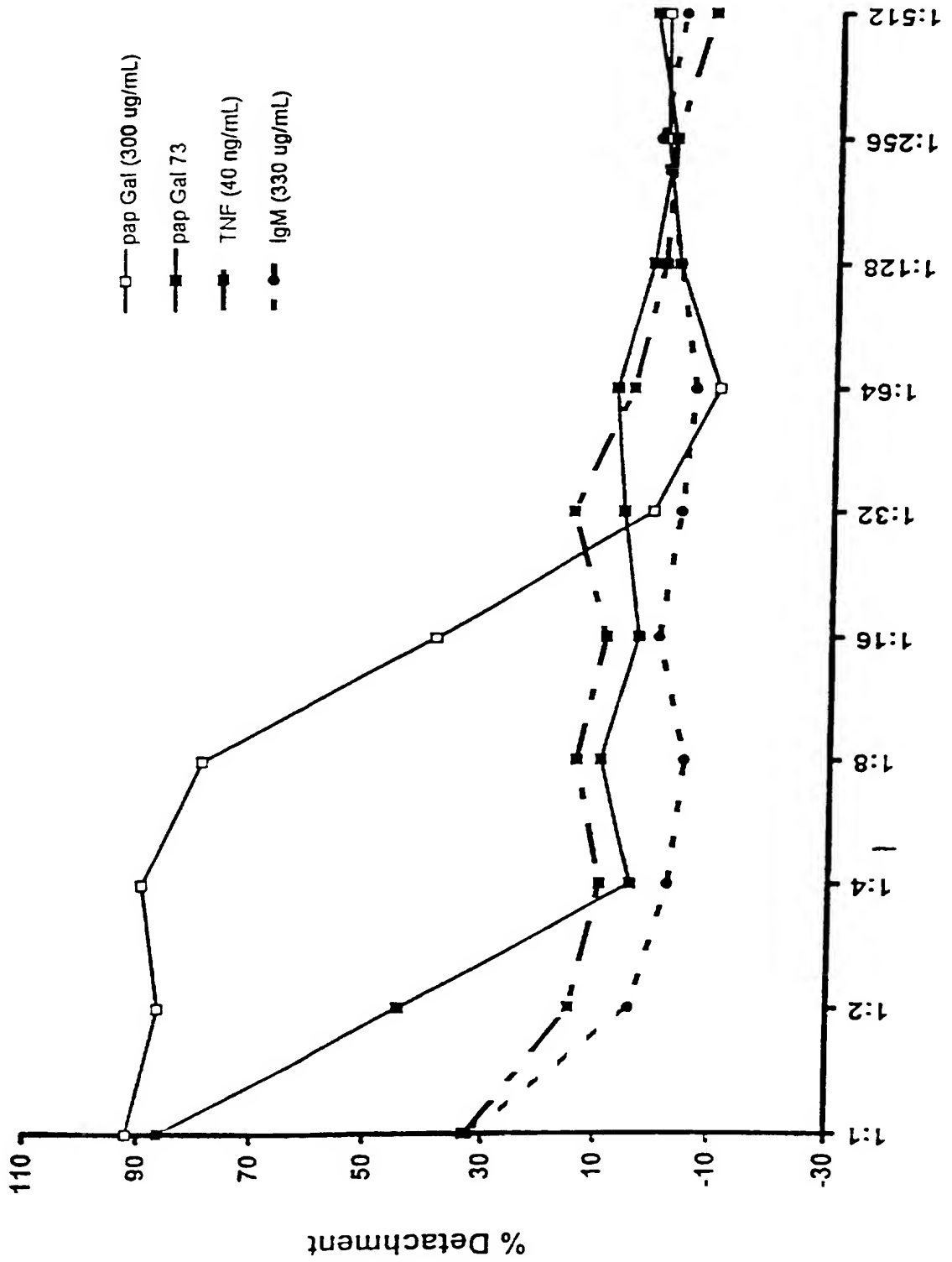
pap Gal (300 ug/mL)
pap Gal 73
TNF (40 ng/mL)
IgM (330 ug/mL)
% Detachment
110
90
70
50
30
10
-10
-30
Dilutions
1:1
1:2
1:4
1:8
1:16
1:32
1:64
1:128
1:256
1:512

FIGURE 10

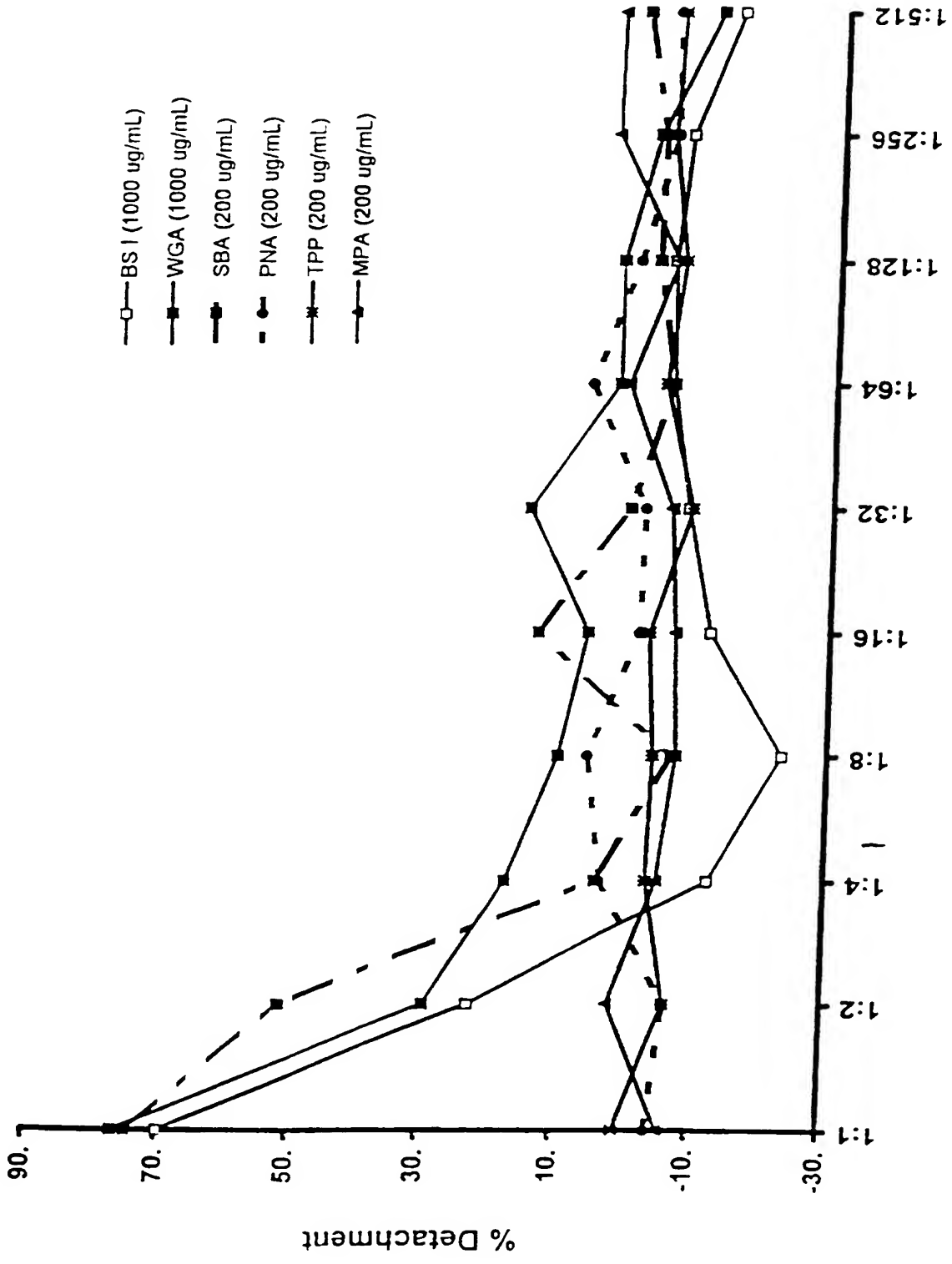
BS I (1000 ug/mL)
WGA (1000 ug/mL)
SBA (200 ug/mL)
PNA (200 ug/mL)
TPP (200 ug/mL)
MPA (200 ug/mL)
% Detachment
90.
70.
50.
30.
10.
-10.
-30.
1:1
1:2
1:4
1:8
1:16
1:32
1:64
1:128
1:256
1:512
Dilutions

FIGURE 11

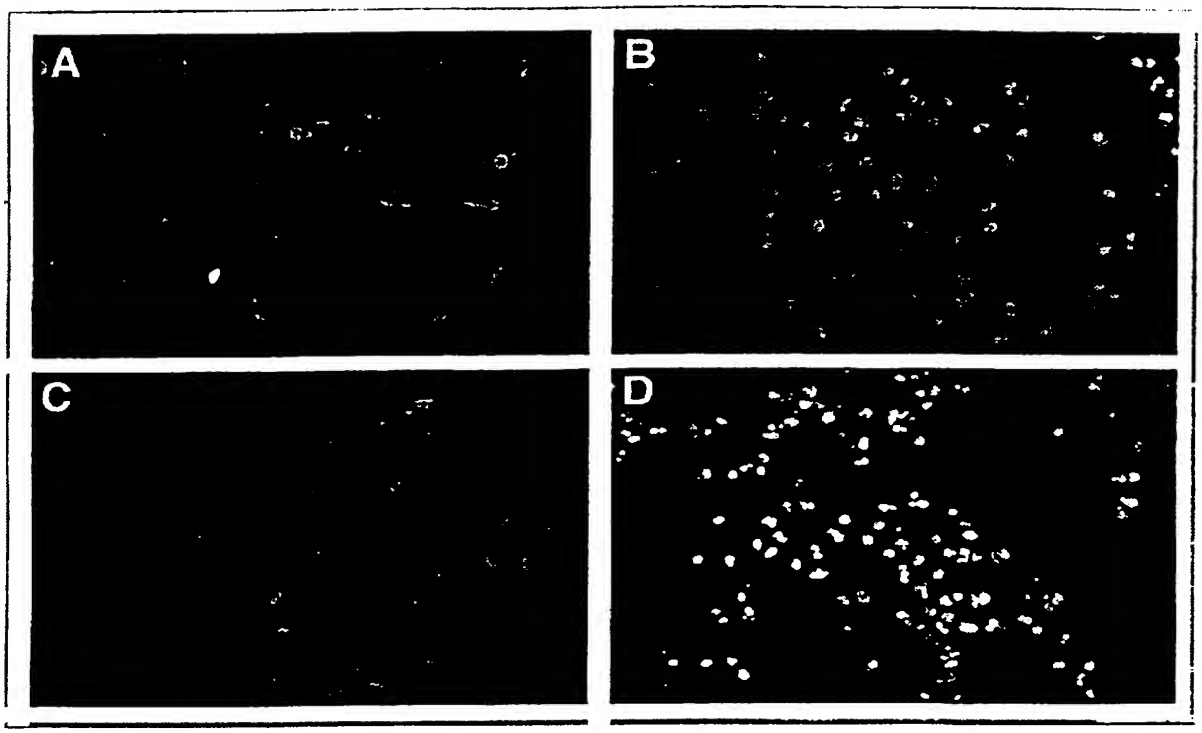


FIGURE 12

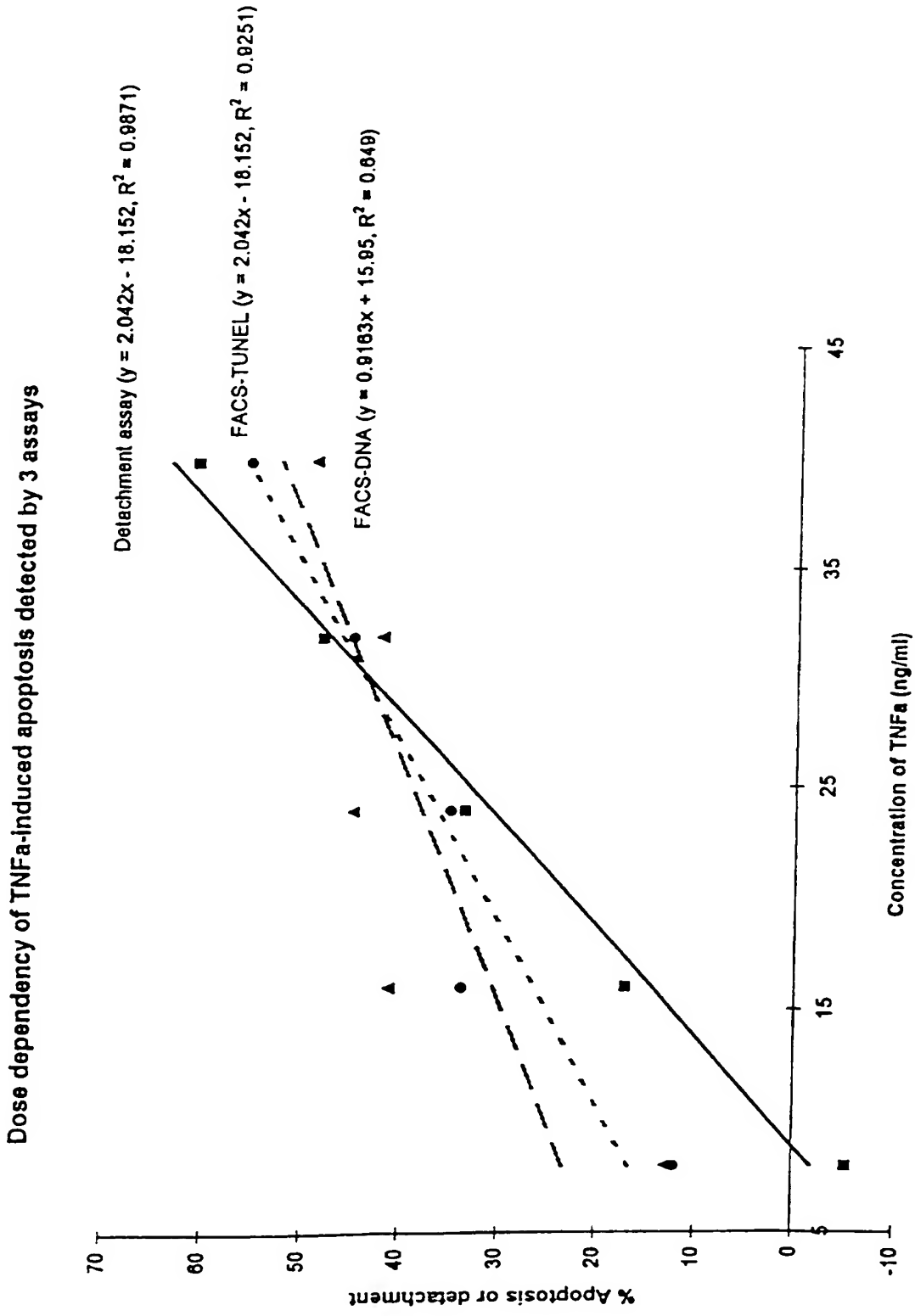
Dose dependency of TNFa-induced apoptosis detected by 3 assays
Detachment assay (y = 2.042x - 18.152, R2 = 0.9871)
FACS-TUNEL (y = 2.042x - 18.152, R2 = 0.9251)
FACS-DNA (y = 0.9183x + 15.95, R2 = 0.849)
% Apoptosis or detachment
70
60
50
40
30
20
10
0
-10
5
15
25
35
45
Concentration of TNFa (ng/ml)

FIGURE 13A

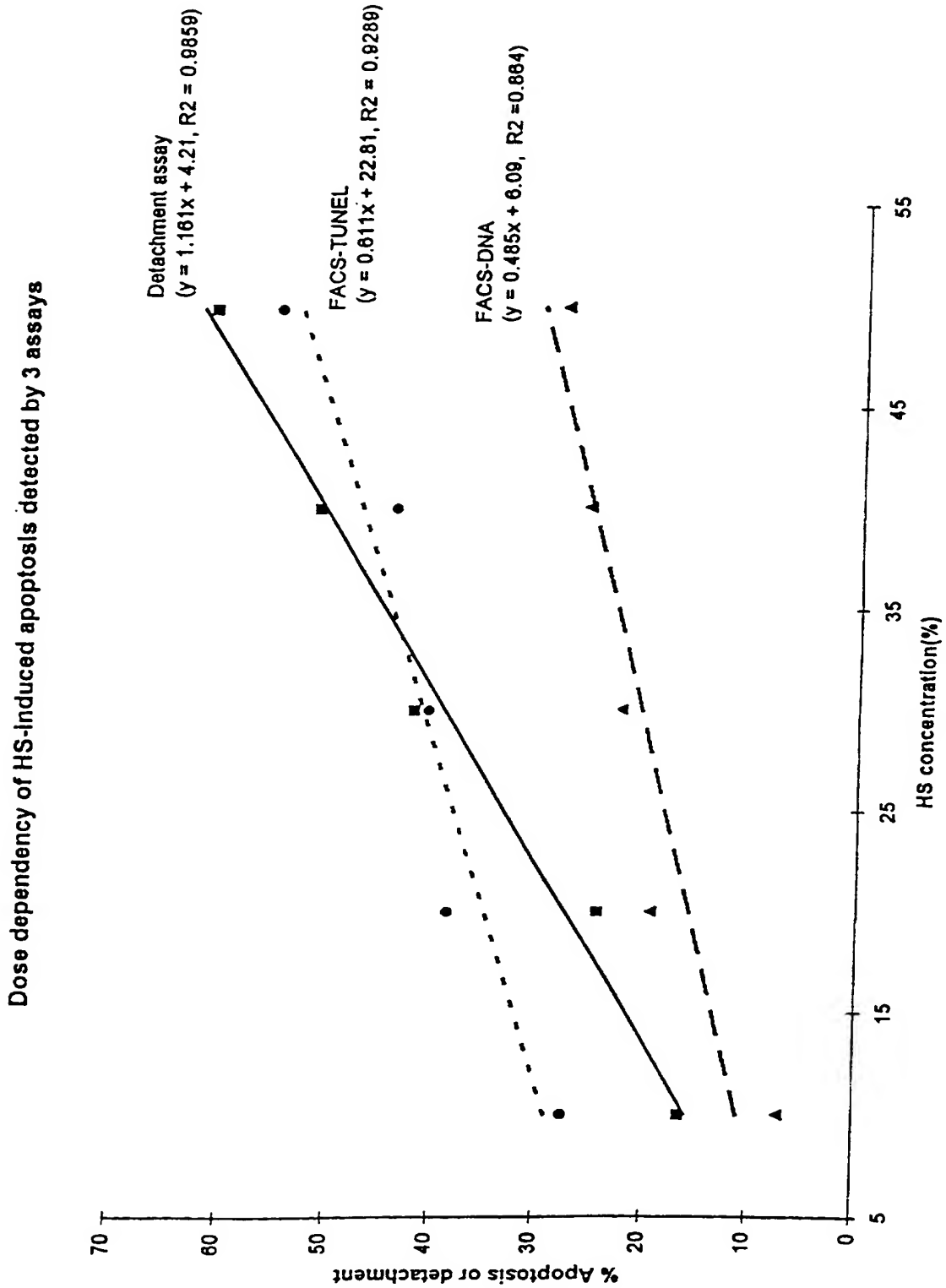
Dose dependency of HS-induced apoptosis detected by 3 assays
Detachment assay
(y = 1.161x + 4.21, R2 = 0.9859)
FACS-TUNEL
(y = 0.611x + 22.81, R2 = 0.9288)
FACS-DNA
(y = 0.485x + 6.09, R2 =0.884)
% Apoptosis or detachment
HS concentration(%)
0
10
20
30
40
50
60
70
5
15
25
35
45
55

FIGURE 13B

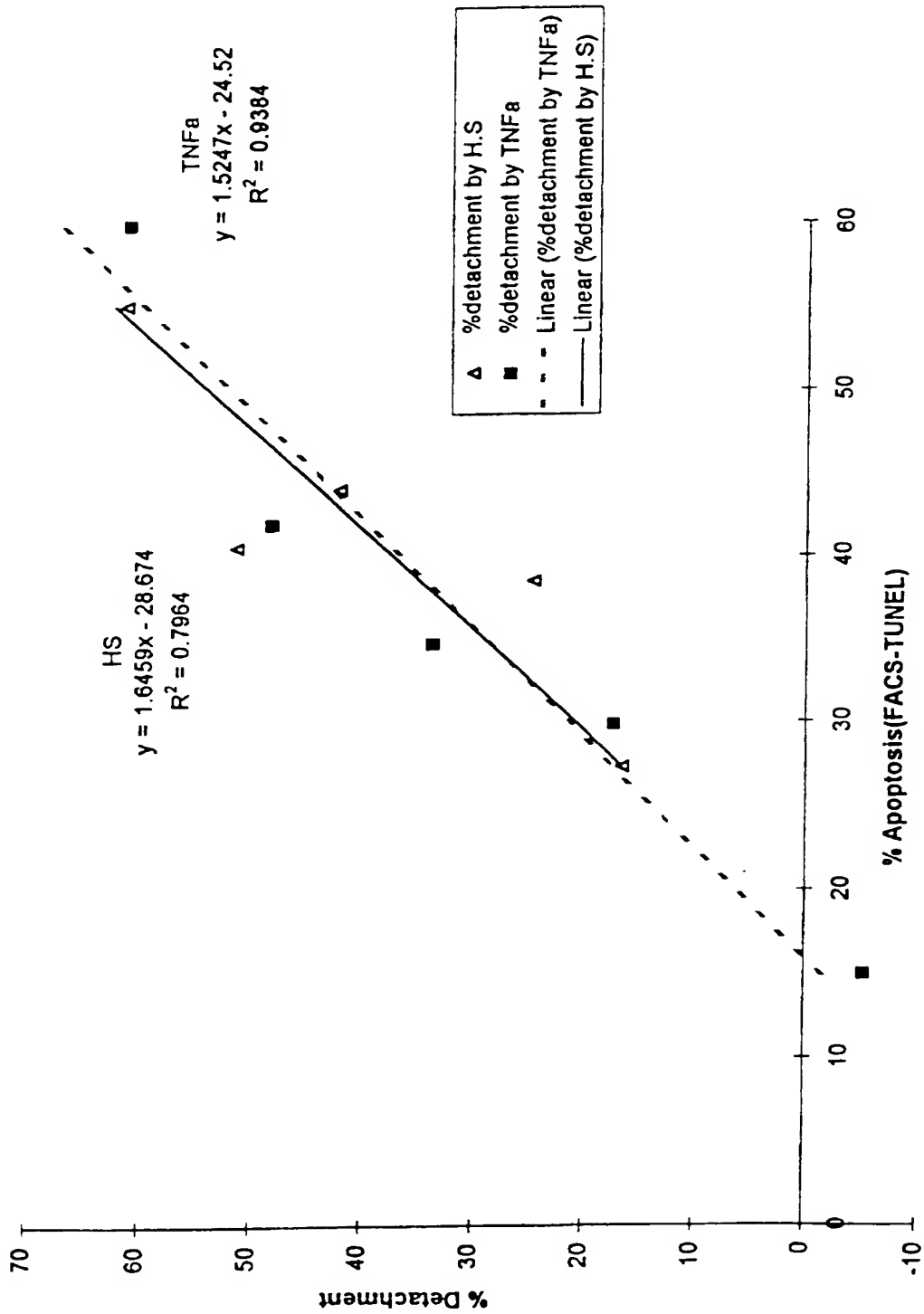
%detachment by H.S
%detachment by TNFa
Linear (%detachment by TNFa)
Linear (%detachment by H.S)
TNFa
y = 1.5247x - 24.52
R2 = 0.9384
HS
y = 1.6459x - 28.674
R2 = 0.7964
% Detachment
% Apoptosis(FACS-TUNEL)
70
60
50
40
30
20
10
0
-10
0
10
20
30
40
50
60

FIGURE 14

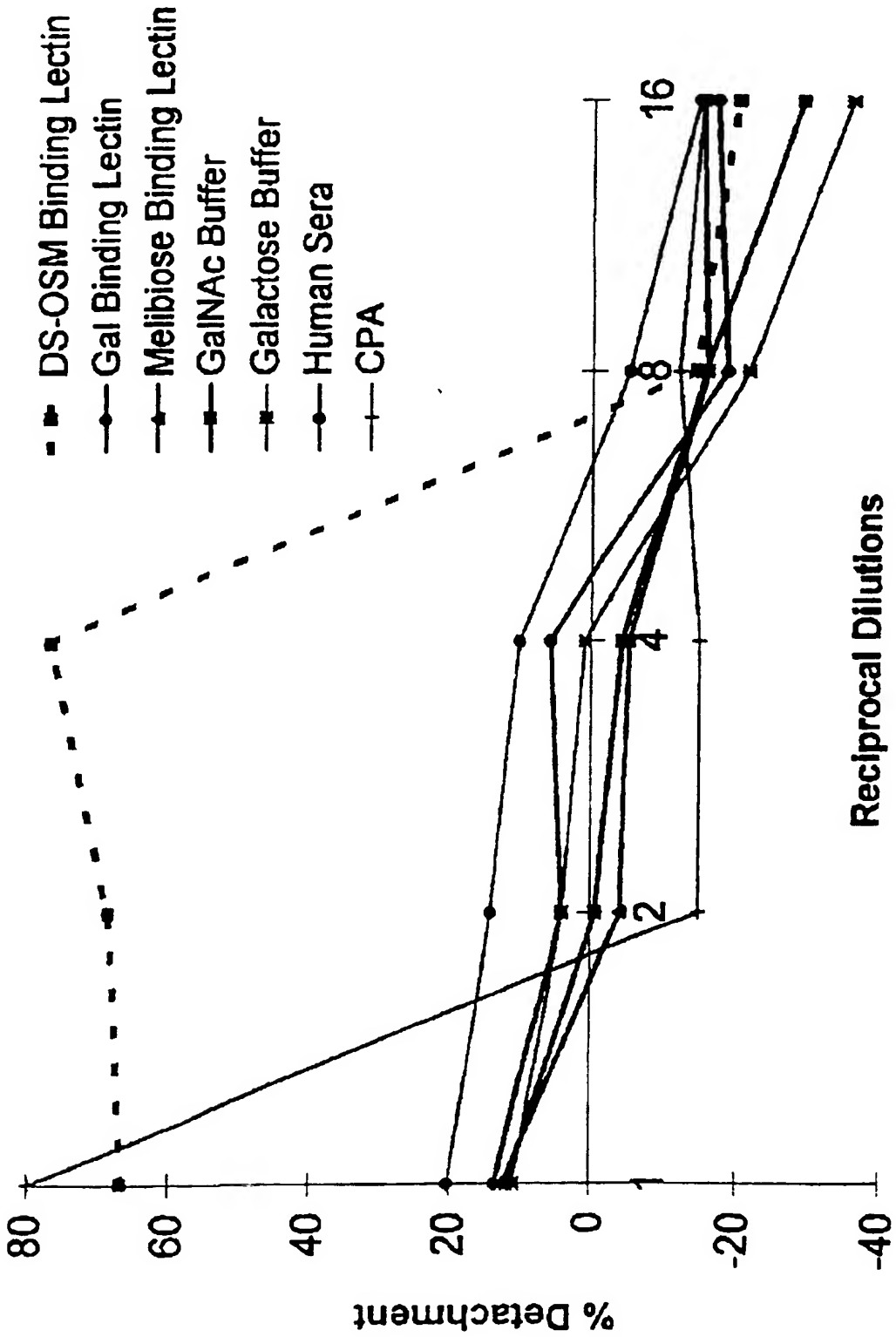
DS-OSM Binding Lectin
Gal Binding Lectin
Melibiose Binding Lectin
GalNAc Buffer
Galactose Buffer
Human Sera
CPA
% Detachment
80
60
40
20
0
-20
-40
Reciprocal Dilutions
16
8
4
2
1

Figure 15

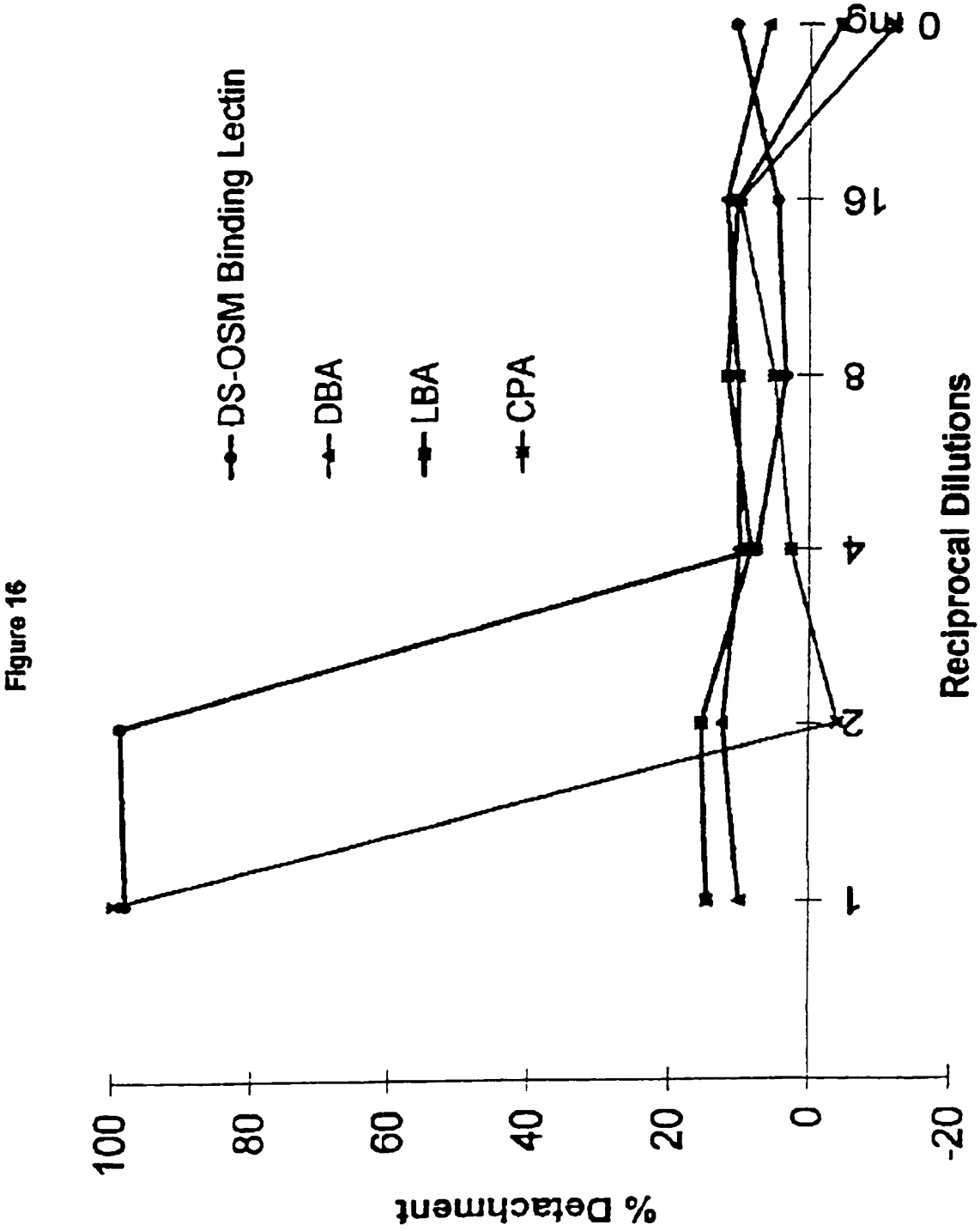
DS-OSM Binding Lectin
DBA
LBA
CPA
% Detachment
100
80
60
40
20
0
-20
Reciprocal Dilutions
0 mg
16
8
4
2
1

Figure 16

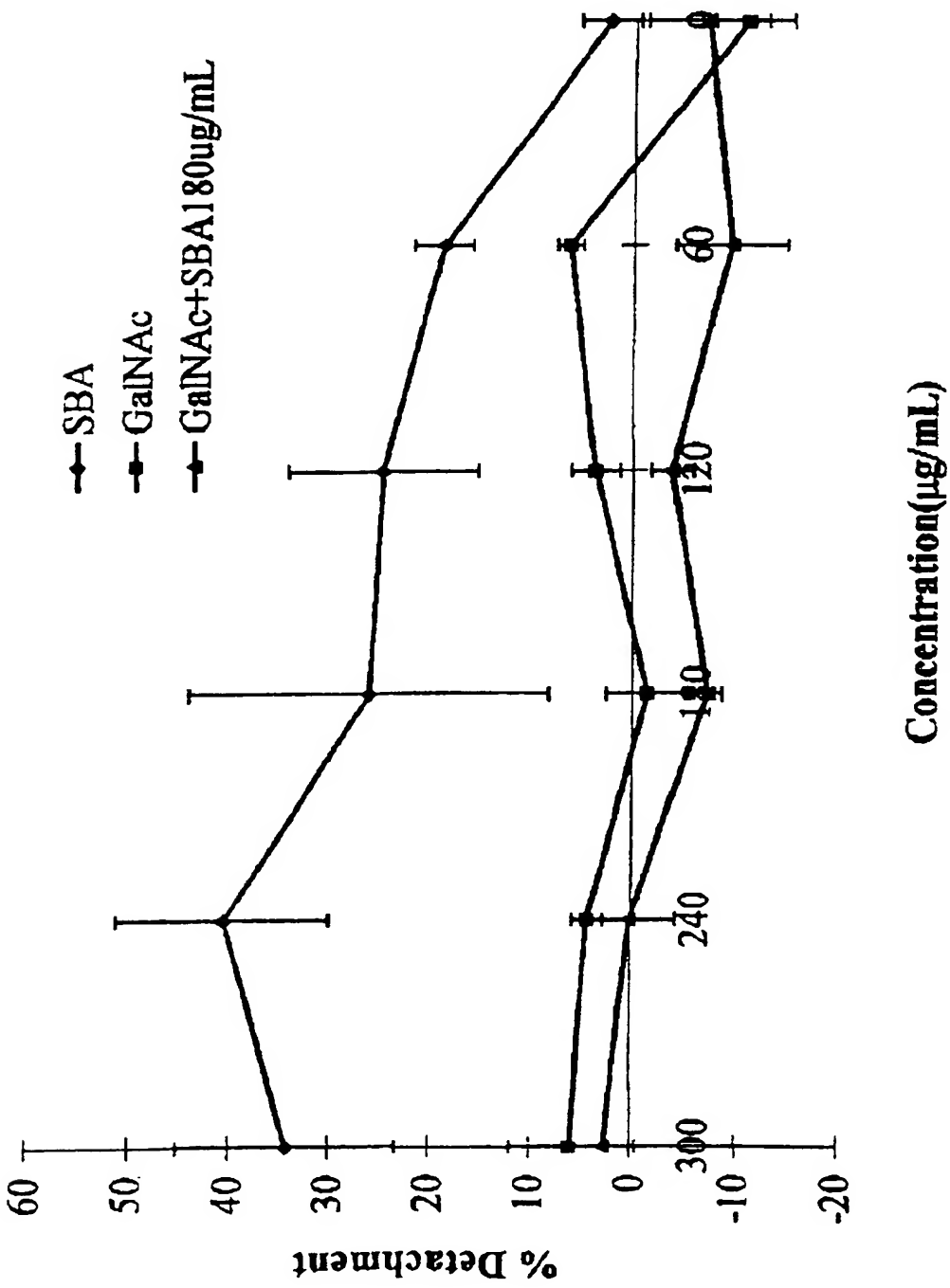
SBA
GalNAc
GalNAc+SBA180ug/mL
% Detachment
60
50
40
30
20
10
0
-10
-20
300
240
180
120
60
0
Concentration(µg/mL)

Figure 17.